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THE MORTALITY FROM CANCER OF THE SKIN AND LIP IN CERTAIN OCCUPATIONS.

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SUMMARY.

THE data given below are derived from an examination of the death certificates of cases of cancer of the skin, lip, penis and scrotum in males from England and Wales for the 34 years 1911 to 1944 inclusive. In the publications of the General Register Office during the period in question (1911 to 1944) cancers of the penis and scrotum were included under the heading "Skin" during 1911 to 1916, and were recorded separately, but under a common heading, "Penis and Scrotum," during 1917 to 1922. In 1923 and subsequently the three forms were recorded under separate headings. For the purposes of this paper, the approximate numbers of deaths from cancer of the penis and scrotum during 1911 to 1916 were ascertained by interpolation from the data for 1917 to 1920. The whole number of certificates examined individually was over 22,000. This study was carried out on the general lines followed in the examination of death certificates of cases of cancer of the lung and larynx (Kennaway and Kennaway, 1936; 1947). The data illustrate the social differences in mortality from cancer of an exposed site which was studied by Stevenson (1923), and subsequently by Conrad and Bradford-Hill (1939).

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The present paper deals with cases of cancer of the skin and of the lip, numbering 5915, in agricultural workers, miners and quarriers, and the professional classes. These three groups numbered 2,612,748 occupied and retired males aged 14 and over at the time of the last census in 1931. The coal miners form an interesting group for comparison with the professional classes, for the miner's daily bath, often obtained under very adverse conditions, secures cleanliness for about two-thirds of the time, while in other respects his skin receives very rough treatment. The agricultural workers, in contrast to the miners, are those most exposed to sunlight, which is known to be a carcinogenic factor. The populations engaged in these occupations, and their classification by the Registrar-General in orders and sub-orders, are shown in Table I. The data for cancer of the penis and of the scrotum will be dealt with in another paper.

TABLE I.—*Classification of Occupations, and Populations Engaged in Them.*

Class of occupation.	Registrar-General's Orders and Sub-orders.	Number of named and coded occupations.		Male population aged 14 and over, including retired. (1931 census.)
Agriculture	Order II	17		1,200,397
Mining and quarrying	Order III			
Coal and shale mines	Sub-order 1	7		939,378
Metalliferous mines and workings	,, ,, 2	4	1,018,049	15,524
Other mines and quarries, brine and oil wells	,, ,, 3	6		63,147
Professional occupations (excluding clerical staff)	Order XXV	33*		394,302
		67		2,612,748
			Occupied	2,439,509
			Retired	173,239

* In Table II this number is reduced to 28 by the pooling of some of the smaller groups.

METHOD OF CALCULATION OF PERCENTAGES.

In judging of the occupational incidence of any form of cancer it is, of course, necessary to correct for age distribution, for an occupation employing a large proportion of older men will yield more cases of cancer apart from any aetiological factors. The census returns (Registrar-General, 1938) give the age distribution, in 5- and 10-yearly periods, of the whole population of males, and also of those following each one of the recognized occupations, at the time of the census. The number of deaths attributed to cancer of the skin and lip occurring in the whole male population in each of these age groups during the years in question was obtained from the Statistical Reviews of the Registrar-General. By applying the death-rates at ages from cancer of the skin and lip in the general male population to the population at the corresponding age-period in each occupation, the calculated or expected number of deaths in the particular occupation can be obtained, and these can be compared with the number actually recorded. Such a comparison is by no means an exact one for skin cancer, because the death certificates dealt with included all cases with mention of skin cancer whether or not the certifier considered it to be the underlying cause of death. Since 1940 some of the deaths have been classified in the Registrar-General's statistics to an associated disease which the certifier thought more important; and even before that date not every death with mention of skin cancer was classified to that cause by the rules then in use. However, as the next section shows, 11 per cent of the certificates used in this study were

rejected for various reasons, so the correspondence between the expected and recorded deaths in different occupations may be regarded as close enough not to invalidate the conclusions reached, though the ratios of recorded to expected are probably in general rather too low.

The populations taken were those of the 1931 census, which is the last census taken, and is near the middle of the whole period in question.

DEATH CERTIFICATES TAKEN FOR EXAMINATION.

The aim of this selection has been to restrict the inquiry as far as possible to primary malignant growths of the epidermis, and of the lip, and to exclude (i) any tumours possibly congenital (e.g. angiomas and melanomas); (ii) any case, the description of which did not exclude sarcoma, nor cancer of an internal site spreading to the skin, nor metastases in lymph glands involving the skin, e.g. "cancer of the thigh," "cancer of the neck and face," "cancer of the face and antrum." Certificates of persons under 14 years of age were excluded. The forms of words in the certificates taken for examination, and in those rejected, may be summarized thus:

(a) *Retained.*—Cancer, carcinoma, epithelioma, rodent ulcer, malignant growth, malignant disease, malignant papilloma, malignant wart, malignant ulcer, or cancerous ulcer, of any part of the skin.

(b) *Rejected.*—(1) Any new growth qualified by "probable," "doubtful," or "query." (2) Sarcoma. (3) Any growth associated with lupus or attributed to X-rays. (4) Adenoma, adenocarcinoma, angioma, naevus, angio-epithelioma, naevo-carcinoma, pigmented or malignant mole, melanoma, dermoid tumour, xeroderma pigmentosum. (5) Any growth of groin, glands of groin, inguinal region or glands, or iliac glands. (6) Cancer, carcinoma or malignant disease of leg, thigh, hip, genitals, throat, neck, glands of neck, face, neck and face, urethra, anus, "pelvis and buttock," "shoulder and head," "epithelioma of neck," where skin is not mentioned.

This selection reduced the number of certificates for the occupations in question from 6665 to 5915, or by 11 per cent.

RESULTS.

1. *Cancer of the Skin.*

(a) *Occupational distribution of total mortality.*

The lower section of Fig. 1 shows the percentages, arranged in ascending order from left to right, for 41 occupations in which more than 5 deaths from cancer of the skin occurred in the 34-year period; this quite arbitrary limit in number of deaths is drawn to lessen sampling error. The percentage in 15 out of 16 professional occupations selected thus is below 100, while that in 9 out of 13 agricultural occupations is above 100, and in 5 is above 150. The percentage in 6 out of 12 mining occupations is above 100.

Table II shows the populations, and percentages, for each of the 67 occupations of the 5 orders and sub-orders (Table I) arranged in descending order of the percentages for cancer of the skin. More than one-half of the professional classes belong to Social Class I; the miners are about equally divided between

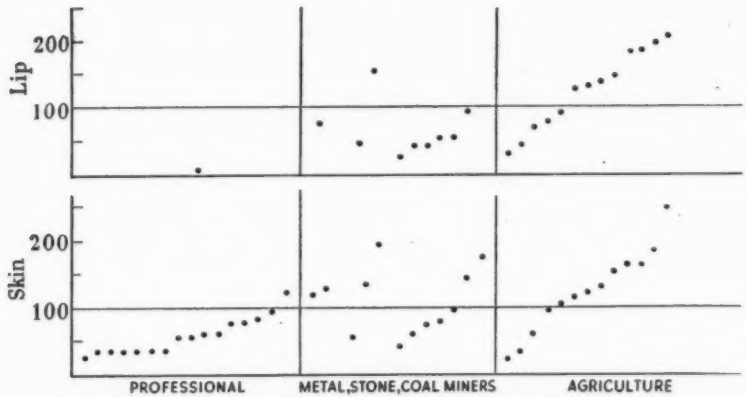

FIG. 1.—Percentages in 41 occupations based on all cases of Cancer of the Lip, and upon cases of Cancer of the Skin numbering more than 5 in any one occupation. Males. 1911 to 1944 inclusive. England and Wales.

Classes III and IV, while the agricultural workers are spread over Classes II to V, with a preponderance of Class IV.

Only one out of 28 professional occupations shows a percentage which is above 100, namely civil engineers and surveyors (121·3), who belong to Social Class I, and this profession appears to show a special liability to cancer of the lip also (p. 11). This occupation (53 deaths) may involve, especially in earlier life, exposure to carcinogenic agents. The occupations of mining engineers, and mechanical and electrical engineers, show no deaths from cancer of the lip, and low percentages for cancer of the skin, but the numbers of deaths (5 and 2 respectively) are small.

The high standard of cleanliness maintained by the coal miner at the end of his day's work, and his lack of exposure to sunlight, perhaps compensate for the various injurious factors, of which an account has been given by Knowles (1944), to which his skin is exposed. First-hand descriptions of the coal-miner's life are given in two recent books (Shaw, 1946 ; Agnew, 1947). But the very much lower incidence upon the professional classes shows that there are still other factors to which the miner is exposed which we cannot yet define. The mortality in the hewers and getters, who carry out much of the most severe work of the mine, is, if anything, lower (97·7 per cent) than it is in the general population. The percentage is below 100 in 5 coal-mining occupations, comprising 828,246 out of the 939,378 men, or 88 per cent of those engaged in the 7 divisions of the industry. The percentage in the whole coal-mining group is 93·2, while in the two other groups it is somewhat higher (metal miners 118·0, other miners and quarriers 101·4), and in all the miners together is 94·4.

The mining occupations offer a wide range of conditions. Some workers (quarriers ; chalk, sand, clay and gravel pit workers) may be exposed to sunlight ; however, the percentages in these groups show a very wide range (17·4 to 194·4). Other occupations (persons conveying material to the shaft in coal mines) may involve contact with lubricating oils, but actually this class of coal miner shows the lowest figure (46·0).

TABLE II.—*Cancer of Skin and Lip. Males. England and Wales.* 1911 *to* 1944. *Populations, Social Classes, and Percentages. The Occupations in each Group are arranged in Descending Order of the Percentages for Cancer of the Skin.*

A.—Professional Classes.

Occupation.	Social class.	Population aged 14 and over. 1931 census.	Calculated deaths.		Total registered deaths.		Registered deaths as % of calculated deaths.	
			Skin.	Lip.	Skin.	Lip.	Skin.	Lip.
Professional engineers (civil engineering and surveying)	I	24,352	43·7	..	53	5	121·3	..
Mental attendants	III	12,361	10·8	..	10	2	92·6	..
Professional engineers (mining engineering)	I	3,086	6·2	..	5	..	80·7	..
Church, chapel, cemetery officials	III	5,810	21·1	..	17	4	80·6	..
Architects	I	10,151	21·9	..	17	1	77·6	..
Judges, stipendiary magistrates and barristers	I	3,632	11·7	..	9	..	77·0	..
Veterinary surgeons and practitioners	II	2,421	6·7	..	5	..	74·7	..
Sick nurses	III	4,106	4·1	..	3	..	73·5	..
Subordinate medical service	II	19,531	29·6	..	18	1	60·8	..
Ministers of other religious bodies	I	11,965	36·6	..	22	1	60·0	..
Laboratory attendants	III	8,001	1·7	..	1	..	58·8	..
Analytical and research chemists and other persons engaged in scientific pursuits	I	18,261	12·4	..	7	..	56·5	..
Painters, sculptors, engravers (artists)	II	10,899	21·4	..	12	..	56·0	..
Dentists	I	11,652	15·5	..	6	1	38·7	..
Roman Catholic priests and monks	I	4,060	7·9	..	3	..	38·0	..
Clergymen, Anglican Church	I	22,760	83·4	..	30	1	36·0	..
Teachers (not music teachers)	II	92,259	147·9	..	52	6	35·0	..
Itinerant preachers, Scripture readers, mission workers	II	4,845	11·4	..	4	..	35·0	..
Solicitors	I	17,853	52·2	..	18	1	34·5	..
Librarians, political association officials, industrial and trade association officials, social welfare workers	II	11,969	23·7	..	8	1	33·8	..
Physicians, surgeons and general practitioners	I	29,911	65·4	..	21	1	32·0	..
Teachers of music	II	5,191	13·4	..	4	..	29·7	..
Authors, editors, journalists, publicists	I	16,670	24·9	..	7	2	28·0	..
Chartered, incorporated, etc., accountants	I	14,567	19·1	..	5	..	26·0	..
Professional engineers (mechanical and electrical engineering)	I	12,089	14·6	..	2	..	13·7	..
Other professional occupations	..	1,378	2·3	..	..	..	..	..
Articled pupils and students	I	13,443	1·2	..	..	..	..	..
Ship designers and surveyors	I	1,079	2·3	..	..	..	..	..
Total professional classes	..	394,302	713·1	319·0	339	27	47·5	8·4

Social Class I	215,531
II	147,115
III	30,278
Other professional occupations	1,378
Total	394,302

TABLE II—(*cont.*).

B.—Mining Occupations.

Occupation.	Social class.	Population aged 14 and over. 1931 census.	Calculated deaths.		Total registered deaths.		Registered deaths as % of calculated deaths.	
			Skin.	Lip.	Skin.	Lip.	Skin.	Lip.
(1) *Coal and Shale Mines*—								
Owners, agents, managers	II	4,583	12·0	5·5	21	..	175·0	..
Other workers above ground	IV	106,549	124·1	55·4	175	54	141·0	97·5
Hewers and getters	III	466,194	512·7	221·6	501	124	97·7	56·0
Subordinate superintending staff	III	37,994	59·1	26·0	46	14	77·8	54·0
Persons making and repairing roads	IV	70,875	115·1	52·2	86	20	74·7	38·3
Other workers below ground	IV	91,425	125·2	55·3	80	24	64·0	43·4
Persons conveying material to the shaft	IV	161,758	54.4	21·2	25	9	46·0	42·5
		939,378	1002·6	437·2	934	245	93·2	56·0
(2) *Metalliferous Mines and Workings*—								
Other workers below ground	III	10,610	17·9	..	23	7	128·5	..
Subordinate superintending staff	III	811	2·4	..	3	1	125·0	..
Other workers above ground and in open workings	IV	3,895	6·7	..	8	1	119·4	..
Owners, agents, managers	II	208	1·8	..	..	..	..	..
		15,524	28·8	12·4	34	9	118·0	72·7
(3) *Other Mines and Quarries*—								
Subordinate superintending staff	III	1,987	3·6	..	7	..	194·4	..
Stone miners, quarriers	IV	33,458	41·4	18·2	57	28	137·7	153·4
Owners, agents, managers	II	2,202	4·7	..	5	..	106·4	..
Other mine and quarry workers	IV	3,146	3·4	*Others* 17·1	2	*Others* 8	58·8	*Others* 46·8
Chalk, clay, sand and gravel-pit workers	IV	15,097	15·3	..	8	..	52·3	..
Slate miners, quarriers	IV	7,257	11·5	..	2	..	17·4	..
		63,147	79·9	35·3	81	36	101·4	102·0
Total mining and quarrying occupations		1,018,049	1111·3	485·0	1,049	290	94·4	59·8

Social Class II	6,993
III	517,596
IV	493,460
	1,018,049

TABLE II—(*cont.*).

C.—Agricultural Occupations.

Occupation.	Social class.	Population aged 14 and over. 1931 census.	Calculated deaths.		Total registered deaths.		Registered deaths as % of calculated deaths.	
			Skin.	Lip.	Skin.	Lip.	Skin.	Lip.
Gardeners	III	233,979	250·7	273·4	625	194	249·3	71·0
(Gardeners and gardeners' labourers)	..	285,343	328·6	307·8	650	205	198·0	66·6
Farm bailiffs	II	10,749	24·2	11·0	45	15	186·0	137·1
Shepherds	IV	11,352	26·6	12·2	69	25	165·4	205·1
Agricultural labourers	IV	361,097	711·4	327·0	1173	598	165·0	183·0
Estate labourers	V	5,496	16·8	7·5	26	11	154·8	146·9
Persons employed in charge of horses	IV	71,616	65·6	28·2	86	52	131·0	184·5
Agricultural machine, tractor proprietors, etc.	III	8,568	11·6	5·1	14	10	120·7	195·3
Farmers	II	260,374	758·1	348·7	861	250	113·6	71·7
Farm foremen	III	7,262	12·5	5·5	13	7	104·0	126·6
Foresters and woodmen	III	13,386	30·5	14·0	30	18	98·4	128·6
Persons employed in tending cattle, dairying, etc.	IV	63,898	56·6	24·4	35	19	61·8	78·0
Land and estate agents, managers	II	2,743	9·1	4·2	4	4	44·0	95·3
Gardeners' labourers	V	51,364	77·9	34·4	25	11	32·0	32·0
Other agricultural occupations	IV	23,872	32·2	14·1	9	6	23·0	42·7
Farmers' sons and relatives	II	72,885	15·6		..		..	
Agricultural and forestry pupils	II	1,711	0·21	5·6	..	..	..	..
Pea and fruit pickers	V	45	0·1		..		..	
Total agricultural occupations	..	1,200,397	2,099·7	1,115·3	2,990	1,220	142·4	109·4

Social Class II	348,462
III	263,195
IV	531,835
V	56,905
	1,200,397

All arithmetical work in this table has been carried to one place of decimals because this seemed worth while when the figures were small. The result, however, is that in the totals there are certain large figures carried to one place of decimals (e.g. 1002·6). One hopes that this will not give an impression that a spurious accuracy is being attempted. Such figures are merely retained for consistency and ease of checking. The sampling error, in the registered deaths column particularly, will affect more than the first place of decimals.

In the agricultural group, the general high level of percentages is clear. Six out of 17 occupations, with a population of nearly 700,000, have percentages from 131 to 249 ; this last figure (gardeners) is the highest of all those recorded in this paper. The contrast between gardeners (249) and gardeners' labourers (32) is obvious. This difference, which appears also in regard to many other diseases, is discussed by the Registrar-General (1938, p. 79, and Table 4D, p. 307), who says, " The separation of agricultural labourers including shepherds from gardeners' and estate labourers, pea and fruit pickers was attempted in No. 93 and 94, Tables 4D, 6A, but the very low mortality figures for Group 94 (gardeners' labourers, estate labourers, pea and fruit pickers) for every cause suggests that the definition of these occupations on death certificates was not as precise as on census schedules, and no reliable conclusions can be drawn from this subdivision." One may suppose that the informant at death registration would often call the deceased a " gardener," when the man himself would have described himself as " gardeners' labourer " at the census. In view of this factor, the result of pooling the figures for the two occupations is shown in Table II ; the percentage for cancer of the skin is then 198 in place of 249.

The figures for the registered deaths as percentages of the calculated deaths for each of the three classes (professional 47·5, mining 94·4, agricultural 142·4) have been included in Table II ; the result, of course, depends largely upon the population of the larger groups, e.g., the figure for mining (94·4) is influenced by the large number (466,194) of hewers and getters (percentage 97·7).

A study of the social distribution of cancer of the skin in a large number of occupations in England and Wales in 1930–32 has been made by Ryle and Russell (1947).

The mortality from cancer of the skin and of the lip upon these groups of occupations may be examined in another way (Table III), namely by calculating the populations which produce one case of cancer in the period in question. (The factors used in calculating the standardized figures for deaths from cancer of the lip shown in Table III were derived from the census population for males over 14 in 1931, and the published figures for deaths from cancer of the lip in the period 1923–1943. The fact that these factors are applied to the occupational deaths in the period 1911 to 1944 is not thought to introduce an appreciable error.) The age distribution of the various groups must be considered, as an occupation employing a large proportion of older people will show a high incidence of cancer apart from any occupational factors. The results given by the standardized and un-standardized figures are shown in Table III. The number of professional workers producing one death from cancer of the skin (1163 ; 1549)

TABLE III.—*Occupational Mortality. Cancer of Skin and Lip.*

Occupational group.	Population. Males, occupied and retired, aged 14 and over. 1931 census.	Deaths, 1911 to 1944. Cancer of		Population producing one death from cancer of skin and cancer of lip.			
				Unstandardized.		Standardized.	
		Skin.	Lip.	Skin.	Lip.	Skin.	Lip.
Agriculture	1,200,397	2,990	1,220	401	878	516 = 1·0	1,590 = 1·0
Mining	1,018,049	1,049	290	970	3,510	780 = 1·5	2,872 = 1·8
Professional	394,302	339	27	1,163	14,000	1,549 = 3·0	20,644 = 12·9
Total of above groups	2,612,748	4,378	1,537	597	1,700	660	2,167
General population	14,050,209	19,087	8,481	736	1,657	736	1,657

is about three times as great as the corresponding figure for agriculture (401 ; 516) in both the standardized and unstandardized series, while the miners take an intermediate position (970 ; 780).

(b) *Occupational distribution according to anatomical site.*

The sites of cancer of the skin named on the death certificates were classified as follows :

Lower limb	Head and neck
Upper limb	Face
Trunk	Ear
	Site not stated.

The subdivision of the data into smaller groups, e.g. fingers, hand, forearm, and so on, might seem to give more information, but the sampling errors would be increased, and in such material the assignment to the smaller sites has not been carried out on any uniform system ; e.g. there would be no certainty that some cancers of the fingers had not been classed as of the hand.

The numbers of cancers at these sites in the three groups of occupations, and their percentage distribution, are given in Table IV and Fig. 2. The inferences which can be drawn from such percentages are, of course, very limited, in view of the effect of each item upon the others. The greatest difference between the

TABLE IV.—*Occupational Distribution of Cancers of the Skin, according to Site.*

	Agricultural occupations.			Mining occupations.			Professional occupations.		
	Deaths.	%.		Deaths.	%.		Deaths.	%.	
Lower limbs	119	4·0		68	6·5		32	9·4	
Upper limbs	398	13·3		50	4·8		15	4·4	
Trunk	105	3·5		65	6·2		33	9·7	
Head and neck	167	5·6		66	6·3		15	4·4	
Face	1524	51·0	73·5	578	55·0	78·1	169	50·0	69·7
Ear	505	16·9		176	16·8		52	15·3	
Not sited	172	5·7		46	4·4		23	6·8	
Total	2990	100·0		1049	100·0		339	100·0	

upper and lower limbs is in the agricultural group, where the former gives the higher figure, which result one might attribute to exposure to sunlight. The percentages of head, neck and face together do not differ very much in the three groups, (73·5, 78·1, 69·7). The face may undergo considerable exposure to sunlight in the professional classes.

A study of the distribution of skin cancer according to site in Northern Ireland, in which a different classification of sites is adopted, has been published by Ryle and Russell (1947).

2. *Cancer of Lip.*

Fig. 1 provides a comparison of the percentages for cancer of the skin, and of the lip, selected and arranged in the same way, except that all cancers of the lip are included and not only those shown by occupation yielding more than 5 deaths. The number of fatal cases of cancer of the lip in the professional classes is so small (27 in 34 years in about 400,000 men) that these have been pooled

without calculation of percentages for the individual occupations; the percentage for the whole group is then 8·4, which is the lowest figure obtained in all the investigations by this method carried out by some of the authors of this paper on cancer of the skin, lung, larynx, bladder and prostate. Fig. 1 and Table II

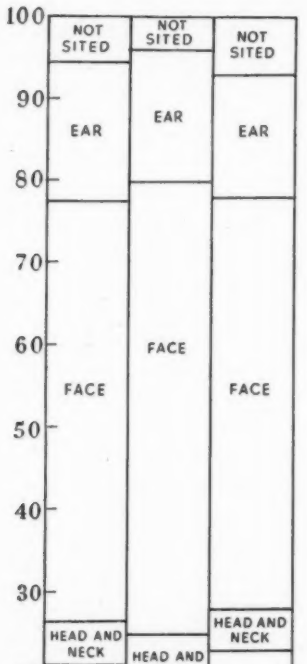

FIG. 2.—Occupational distribution of cancers of the skin according to site.

show that no one of the seven groups of coal and shale miners gives a percentage above 100; the figures are low (38·3 to 56·0 per cent) except in one large group which is the most exposed to light (other workers above ground, population 106,549), in which the incidence (97·5 per cent) is approximately the same as in the general population. The numbers in the other branches of mining (metal miners, other mines and quarries) are small for statistical purposes, but one notes that stone miners and quarriers, many of whom must be exposed to sun-

light, are the only miners showing a percentage (153·4) which is above 100, and that this figure is of the order of that found in open-air workers. Eight of the 13 agricultural occupations represented in Fig. 1 show percentages above 100, the highest figure being 205 (shepherds).

The ratios in the last two columns of Table III show the much greater difference between the professional classes, and the miners and open-air workers, which is revealed by the figures for cancer of the lip in comparison with those for cancer of the skin.

The high incidence of cancer of the lip upon civil engineers and surveyors, in comparison with other professional workers, is shown by the following rough calculation, though the numbers are of course very small (Table V):

TABLE V.—*Cancer of the Lip in Civil Engineers and Surveyors.*

Professional Occupations. Social Class I.

	Population.	Deaths from cancer of lip.	Population producing one death.
Civil engineering and surveying	24,352	5	4,870
Other professions	191,179	8	23,874
	215,531	13	

DISCUSSION.

Cancer of the skin.

The material summarized in Tables II and III, and in Fig. 1 shows that the incidence of fatal cancer of the skin is high upon agricultural workers, and very low upon the professional classes, while the miners take an intermediate position not far removed from that of the general population. The especial liability of the out-door workers is generally supposed to be due to exposure to sunlight (Blum, 1948).

The results given in Table IV and Fig. 2 show that the cancers of the whole region of neck, head, and face together, selected on the basis described above, make up from 70 to 78 per cent of cancers of the skin in all three occupational groups. The face is the only part of the body which is never clothed, and those parts which are shaved receive more thorough cleansing than do any others; these characters are common to all social classes. The most notable result of this tabulation is the lack of any great difference between the three groups.

Another factor which may be of considerable importance is that the cosmetic effect of cancer of exposed portions of the skin and of the lip may attract attention sooner in richer subjects. Ingram (1947), in a comment on the paper by Ryle and Russell (1947), says: "Prognosis in skin cancer depends largely upon the size of the lesion, and negligence is probably an important factor in relation to death from this cause. I should, in the ordinary course of events, expect neglect of a symptomless lesion to be higher in unskilled workers and labourers (IV and V of the Registrar-General's social classes) than in the other groups." Obviously one cannot assess the quantitative importance of this difference.

Data in equal detail for the distribution of skin cancer in married women whose husbands follow these occupations would be of great interest. The Decennial Supplement for 1931 shows that the comparative mortality from

cancer of the skin upon the Registrar-General's five social classes is similar in men and married women (Table VI), and the same phenomenon is shown by

TABLE VI.—*Social Distribution of Cancer of the Skin Among Men and Married Women.*

Cancer of Skin, 1930–1932. Standardized Mortality Ratio.

Decennial Supplement, 1931.			Ryle and Russell.		
	Age 35 to 65.			Age 20 and upwards.	
Social class.	Males.	Married women.	Group.	Males.	Married women.
I and II	72	92	A	70	75
III	95	89	B	102	116
IV	116	114	C	160	152
V	132	131	..	..	..

3 groups of occupations, A, B and C, showing a similar descent in the social scale, which were studied by Ryle and Russell (1947), using the Registrar-General's figures.

Such material provides a most valuable indication of environmental, as distinct from occupational, factors. For a discussion of this matter see p. 3 of the Decennial Supplement, 1931, Part II*a*. The Registrar-General (p. 46) concludes that in the case of cancer of the skin, " . . . most of the differentiation arises from selective factors or from environmental conditions in the homes rather than from the occupations themselves . . . although the employment of a proportion, largest no doubt in Classes IV and V, of married women in textile occupations may be partly responsible for the mortality gradient for skin cancer."

Cancer of the lip.

Material obtained from death certificates does not allow one to distinguish between cancers arising from the upper or lower lip, or from different portions of either of these. For a discussion of aetiological factors see Lane-Claypon (1930), and Willis (1948), who says (p. 294) ". . . The great preponderance of tumours of the lower lip is explained as due to direct exposure of its muco-cutaneous junction to sunlight, while the corresponding part of the upper lip is relatively shaded." Some factor in addition to exposure to sunlight is required to explain the differences shown in Table VII. Smoking, and especially pipe smoking, is, of course, regarded as another possible factor in the production of cancer of the lip. During the earlier part of the period (1911 to 1944) cigarette smoking was practically restricted to the richer classes, which produce fewest cancers of the lip ; data on the change in this matter in recent years have been given elsewhere (Kennaway and Kennaway, 1947). The data collected by Lane-Claypon (1930) from the literature, summarized in Table VII, show the great preponderance of cancers of the lower lip, especially in males. Of 4839 cases where the sex was stated, 93·5 per cent were male. Of 3763 cases where the site was stated, the lower lip was involved in 94·2 per cent. Table VII records the data from 2676 cases in which sex and site for each individual were stated.

Lane-Claypon's data are derived from published series, which might be liable to some kind of selection, but the greater incidence upon males is con-

TABLE VII.—*Cancer of the Lip* (*Lane-Claypon*, 1930).

	Males.		Females.		
	Number.	%.	Number.	%.	% of Total.
Lower lip	2378	96·4	160	76·2	6·3
Upper lip	88	3·6	50	23·8	36·2
Totals { Male	2466	100	210	100	7·8
Totals { Female	210				
	2676				

firmed by the unselected figures of the Registrar-General. In the years 1923–43 inclusive there were in England and Wales 5897 deaths attributed to cancer of the lip, of which 506, or 8·6 per cent, were in women.

The high incidence of cancer of the lip upon outdoor workers is shown by figures for England and Wales for 1911 to 1913, obtained by Lane-Claypon (1930) from Dr. Stevenson of the General Register Office (Table VIII).

TABLE VIII.—*Cancer of Lip. Males. England and Wales*, 1911–13. (*Lane-Claypon*, 1930).

	Deaths.	Death rate per million per annum.
All occupied and retired males	750	21·2
Agricultural labourers	128	79·8
Farmers	56	55·0

Comparison of cancer of the skin and of the lip.

The data given in Fig. 1 and Table II, and especially in the two last columns of Table III, show differences between the three occupational groups which are greater in the case of cancer of the lip than in that of cancer of the skin. These results, derived from certain selected occupations, may be compared with those given by the Registrar-General for the social distribution of these cancers in the whole male population (Table IX), which show a much steeper social gradient in the case of cancer of the lip than in that of cancer of the skin. The results

TABLE IX.—*Comparative Social Incidence of Cancer of the Skin and of the Lip.* (*Registrar-General*, 1927, 1938.)

Standardized Mortality Ratio.*

	Males. England and Wales.				
Social class	I.	II.	III.	IV.	V.
	Ages 20–65. 1921–23.				
Cancer of skin	63	73	100	120	150
Cancer of lip	30	50	70	140	170
	Ages 35–65. 1930–32.				
Cancer of skin	†(59)	75	95	116	133
Cancer of lip	†—	56	68	147	183

* Figures for 1921–23 are based on those for all occupied and retired males, while those for 1930–32 are based on those for all males (i.e. including unoccupied).

† Ratios based on less than 20 registered deaths are shown in brackets, or if both the registered and standard deaths are less than ten, omitted.

reported in the present paper are an attempt to carry this analysis further by a study of occupations providing different environmental conditions. The ratios of the populations producing one death from cancer of the skin, or of the lip (Table III), namely,

	Agriculture.	Mining.	Professional occupations.
Cancer of skin	1·0	1·5	3·0
Cancer of lip	1·0	1·8	12·9

indicate that in cancer of the lip, some anti-carcinogenic factors, very much more powerful than the protection from sunlight which the miner's life affords, are active among the professional classes. Ingram's "negligence factor" (see above) must be borne in mind, and also the susceptibility of the lip to defective supply of vitamin B_2 (riboflavin).

The predominantly high incidence of cancer of the skin upon agricultural workers is shown by the fact that these occupations employ 1,200,397 men, and of these, 1,021,857, or 91 per cent, follow occupations in which the percentage for skin cancer is above 100, while the corresponding population in the case of cancer of the lip is 489,526, or 43 per cent. This difference is due to the low incidence of cancer of the lip on the large populations of gardeners and farmers (total 545,717), and it is noteworthy that these occupations show the greatest difference in the incidence of these two forms of cancer. Of the 14 agricultural occupations which produce any cancers of the lip, 11 show agreement in regard to cancer of the skin and of the lip in that both percentages are either below, or above, 100. This suggests that the aetiological factors are to some extent the same in some occupations. The 3 occupations showing discrepancy in this respect are :—

	Registered per cent of calculated deaths.	
	Cancer of skin.	Cancer of lip.
Gardeners and gardeners' labourers	198·0	66·6
Farmers	113·6	71·7
Foresters and woodmen	98·4	128·6

Registered deaths as percentages of calculated deaths for skin cancer in the three main occupational groups considered (professional, mining, agricultural) are fairly evenly spread about the figure of 100 (47·5, 94·4, 142·4), but the corresponding percentages for cancer of the lip are much lower (though in the same order) (8·4, 59·8, 109·4). The fact that the highest of these is 109·4 raises the question, "What are the other occupational groups which show a percentage of over 100 for cancer of the lip?" The investigation will be pursued in this direction.

SUMMARY.

A study has been made of the death certificates of cases of cancer of the skin, and lip, in males engaged in agriculture, mining and professional occupations in England and Wales during 1911 to 1944. The mortality is highest in agricultural workers, and lowest in the professional classes, while the miners take an intermediate position. The special liability of outdoor workers to cancer of the skin and lip is generally attributed to exposure to sunlight, but the

data given here show that other factors are involved also. The incidence upon the coal miner is of especial interest in view of the cleanliness secured by his daily bath, and of his minimal exposure to sunlight. In occupations in which 88 per cent of the coal-mining population are engaged the liability to cancer of the skin is less than it is in the general population. Only one occupation of Social Class I (civil engineers and surveyors) shows a mortality from cancer of the skin higher than that in the whole population; hence these results illustrate the social distribution of cancer of exposed sites discovered by Stevenson. The comparative distribution of cancer upon the various parts of the skin does not show any great differences in the three groups; from 70 to 80 per cent of these cancers are situated on the head and neck. The difference between the professional classes on the one hand, and the miners and agricultural workers on the other, is much greater in the case of cancer of the lip than in that of cancer of the skin. The agricultural occupations in which the ratio of registered to calculated deaths is greater than 1 contain 91 per cent of the population in the case of cancer of the skin, and 43 per cent in that of cancer of the lip. Only 27 deaths from cancer of the lip were recorded in 34 years in men of the professional classes, who number about 400,000. Various aetiological factors are discussed.

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THE TREATMENT OF RETINAL GLIOMA.

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MARTIN and Reese (1936, 1942, 1945) were the first to describe an extensive series of cases of retinal glioma treated by deep X-ray therapy. In their latest paper they report 75 per cent 5-year cures in 8 cases of bilateral disease, one eye having previously been removed by surgery. Four of the 6 surviving cases were blind. At the Middlesex Hospital 19 cases of glioma retinae were seen between 1929 and January, 1946. All cases were treated, 4 by surgery alone; in the others radiotherapy of one type or another played a major part in the treatment. The numbers are too small to allow comparisons to be made between different types of treatment, but as regards deep X-ray therapy certain conclusions have emerged, and a technique has been evolved which differs in some respects from that advocated by the workers of the New York Memorial Hospital.

The results of surgery have greatly improved in the last 30 years according to Ray and McClean (1943). Collecting their figures from the literature they estimate that between 1868 and 1941, 18 per cent cures were obtained by enucleation in 677 cases, whereas 50 per cent cures are claimed for the 105 cases so treated between 1911 and 1941. This progress is ascribed to the more radical and improved technique, often with partial excision of the optic nerve, used in later years. The number of bilateral cases included, if any, is, however, not stated. The authors themselves advocate a combined intracranial and orbital operation in view of Reese's (1931) statement that 52 per cent of 119 cases showed invasion of the optic nerve beyond the lamina cribrosa.

Duke-Elder (1940) also states that 50–57 per cent of unilateral cases may expect to be cured. Reese (1940) cites statistics of 95 cases collected by the Division of Ophthalmic Pathology of the American Registry at the Army Medical Museum, 26 of which had bilateral disease. At the time of review 21 cases were untraced, 39 had died, 13 had lived 5 years, 2 had lived 3–4 years, 10 between 1 and 3 years, and another 10 just 1 year.

Besides X-ray therapy, which is historically reviewed by Martin and Reese (1936, 1942, 1945), attempts have been made at treatment of the second eye in bilateral cases by Radon-seeds. These are usually introduced either into the growth itself or sown on to the sclera over the neoplasm. Stallard, in his Gifford Edmond Prize essay (1933), reports 5 cases treated primarily by these methods. One of his cases—first reported on by Moore (1930, 1935), was treated in 1929 (twice by the insertion of a seed into the growth and also by episcleral seeds), is still alive, but has a cataract. Three other cases, including an advanced case treated only experimentally and intended for subsequent histological investigation, came to excision. In a further report Stallard (1948) gives the result of 15 cases so treated. Of the 12 cases treated more than 5 years ago 9 have been followed up from 7–14 years; 6 have sufficient vision to allow of normal

schooling. This method has also been used successfully in a case described by Philps and Houlton (1944), who inserted a single 2·5 mc. seed into the growth for a fortnight. Three and a half years later the boy was well and his vision 6/12.

Spontaneous cures have been recorded in a number of cases (Martin and Reese, 1936, 1942, 1945), and a rather remarkable series in one family by Hine (1937).

Classification.

Of the 19 cases 8 were unilateral and 11 had bilateral involvement. The high proportion of bilateral cases in this series is easily explained by the fact that more of these would be referred for radio-therapy than of the unilateral cases.

There were 4 cases of recurrence after an attempt at cure had been made elsewhere, and of the remaining 15 cases, 8 had had an enucleation elsewhere and were referred for radiotherapy.

TABLE I.—*Primary Cases.*

	Previously untreated.	Referred after enucleation of one eye.		Total.
		For post-operative radio-therapy to orbit.	For radio-therapy to 2nd eye.	
Unilateral	4	3	—	7
Bilateral	3	—	5	8*
Total	7	3	5	15

* Both eyes were affected simultaneously in all but one case, where the second growth in the right eye was not seen until 3½ years after removal of the left eye.

Sex.—13 of our cases were male, 6 female children.

Age.—Ages at the time of treatment varied from 7 months to 5 years.

TABLE II.—*Age.*

	Years.					
	0–1.	1–2.	2–3.	3–4.	4–5.	5–6.
(*a*) At time of treatment (primary cases)	2	3	6	1	2	1
(*b*) At onset (unknown in 5)	8	3	2	1	—	—

The length of history before the primary treatment, where determined (unknown in 7), is shown in Table III.

TABLE III.—*Length of History.*

Months.				Years.
Under 1.	1–2.	4–6.	6–12.	18.	2–2½.
1	3	3	3	1	1

It is therefore seen that in a high proportion of cases there was considerable delay in starting treatment.

Side affected.—In all but one of the unilateral cases the right eye was affected, whereas in 9 of 11 bilateral cases the condition was more advanced in the left eye, and this was the eye excised, the other to be treated by radiotherapy. This is a rather remarkable antithesis, and unlikely to have arisen by chance.

Family history.—A positive family history was obtained in only one case in this series—a girl of 2½ years with bilateral disease whose elder sister had had an eye removed for retinoblastoma. Since then we have seen a boy of 13 months with very advanced bilateral disease, whose father had lost both eyes through congenital buphthalmos. Most of our cases, however, belong to the sporadic variety, in which bilateral incidence is rare as a rule.

Pathology.

The bulk of the histology of our cases was carried out at the Royal London Ophthalmic Hospital and the Bland-Sutton Institute of Pathology of the Middlesex Hospital, to whose directors my thanks are due for their permission to use these records.

TABLE IV.—*Histological Type.*

Neuro-epithelioma	3
" Retinoblastoma " or " glioma retinae " . . .	16

TABLE V.—*Optic Nerve Involvement.*

Present.	Absent.	Not stated.
4	8	7

These tables show that only a small percentage of our cases fall into the relatively more benign group of neuro-epithelioma (Parkhill and Benedict (1941), who report " good " results in 10 of 16 cases of this type (62·5 per cent) as against 2 of 16 (12·5 per cent) in retinoblastoma), but on the other hand there were few cases with optic nerve involvement.

Griffith and Sorsby (1944) have expressed the belief that hereditary retinoblastoma is a distinct histological entity, differing from the sporadic type, and Cummings and Sorsby (1944) have shown that in their series the unilateral tumours arose from the outer—whereas the bilateral tumours originated from the inner, nuclear layer or from both layers. The hereditary cases, being bilateral, followed this rule.

Pathological findings in cases ending fatally.—Two cases died outside our hospital service with signs of multiple secondaries in the brain. Two other cases were observed by us during their last stage, and post-mortems were performed, revealing *inter alia* a haemoperitoneum due to rupture of the liver affected by metastases and involvement of the Sylvian aqueduct. (Case Reports 3 and 14.)

Treatment.

A broad outline of treatment as it has been given in the various types of cases will be followed by a more detailed description of the methods of treatment used and a discussion of their relative value in the control of glioma retinae.

TABLE VI.—*Methods of Treatment.*

	Primary.		Recurrence.	
	Unilateral.	Bilateral.	Orbital. (After enucleation.)	Intra-ocular.
Surgery alone	3	1	—	—
Surgery (one eye) + radiotherapy (post-operative or 2nd eye) .	3	7	—	—
Radiotherapy alone . . .	1	—	1	3

A. *Primary cases.*

I. *Unilateral disease.*—Surgery alone was used in 3 cases, one of which showed optic nerve involvement. In 3 further cases surgery was followed by post-operative irradiation, in two of which there was evidence of extrabulbar spread. One case was primarily treated by radiation.

II. *Bilateral disease.*—The eye in which the disease was more advanced was excised in all 8 cases. The other eye was treated by radiotherapy—external radiation being used—in 7 of these. The remaining case was referred to us after the removal of one eye, for radiotherapy to the second eye. The disease even in this eye, however, had progressed so far that the sight could not have been saved; it was therefore decided to enucleate also this, the remaining eye.

B. *Recurrent cases.*

All the 4 recurrent cases were treated by radiotherapy. One case was seen with a recurrence in the socket 5 months after removal of the only affected eye. The other 3 cases had recurrences in the remaining eye following treatment respectively by insertion of radon-seeds into the eye, the insertion of radium needles into the orbit, or of diathermy puncture of the growth, the first eye having been excised.

METHODS OF RADIOTHERAPY.

Prior to 1941 radium was used in our series as the source of irradiation; since then all cases have been treated by deep X-rays.

"*Primary*" *treatment.*

I. *Radium.*—Radium in the form of surface moulds was used in 2 cases for the treatment of the remaining eye. The dosage was calculated in mg.-hours, and has, for the purposes of this investigation, been recalculated—as far as was possible from the data available—in roentgens. It is interesting to note that the tumour dosage then applied was very similar in range to that used later in the X-ray-treated cases.

Two of the 3 bilateral cases who have survived 5 years were treated by this method. The actual survival times are 16 and 11 years. One case was treated on 3 occasions within 6 months, estimated tumour doses of 1440, 2900 and 3800 r. being applied until the growth was obviously shrinking. This boy can read large print. The case was previously reported by Mr. R. A. Greeves (1934). The other case was given an estimated tumour dose of 3000 r. (surface dose 5600 r.), and developed a posterior polar cataract, which is discussed below.

2. *Deep X-ray therapy.*—As long as radium moulds were being used it was inevitable that the whole of the globe was being irradiated to a high dose, but with the change-over to and use of small beams that were developed in deep X-ray therapy it became possible to limit the volume of high dose to a certain extent. Our technique has been directed at eradicating the focus of disease present rather than treating the whole of the posterior half of the retina (where the majority of the growths are situated). A recent case, however (not included in this survey), showed us that a certain risk is run thereby in that shortly after the treatment and subsequent disappearance of a small lesion below the equator by fields of 2 and 3 cm. diameter a further nodule appeared in the upper half of the retina.

In an endeavour to treat as small a part of the eye as possible we have used circular fields of 2 and 3 cm. diameter by means of special tubular applicators primarily intended for intrabuccal lesions as described by Roberts and Quick (1943). These are used in connection with our deep X-ray tubes, treatment being carried out at 190–220 kv. and focus-skin distances of 30 to 35 cm. A Thoraeus filter (1·0 mm. Al + 0·4 mm. Sn + 0·8 mm. Cu), giving a $\frac{1}{2}$-value layer of 2·1 mm. Cu, was used until 1942, since when, except in one case, filtration has been 0·5 or 0·8 mm. Cu, giving a $\frac{1}{2}$-value layer of 1·3 or 1·4 mm. Cu. Multiple fields are used, e.g. lateral (temporal), medial (transnasal), superior and inferior orbitals. The anterior field was abandoned as soon as it became apparent that the cornea was the most vulnerable structure of the eye, and disintegration of the eye had occurred in 2 or 3 cases in which this approach had been used. The lens, too, will receive a relatively large dose of irradiation when the anterior field is used and the lens dose should be kept at a minimum. We endeavour to ensure this by our technique of " by-passing " the lens as much as possible, which can be achieved in all the other fields mentioned.

Positioning.—Special problems of set-up arise because of the age of the patients and the small fields used so that accuracy of alignment is of much greater importance. Most of the children under the age of 4 need steadying during each treatment, and the older ones at least during the first 2 or 3 sessions. We have in a few cases tried to make individual plaster casts into which the child is put during treatment, the cast including a headpiece with a frontal process to obviate any possibility of head movement. In practice, however, even the smallest children have been found to wriggle around in these casts and human hands have had to help. The casts are useful, nevertheless, for steadying at least the trunk, and one escapes the otherwise necessary manoeuvre of binding the child on to the couch. In smaller children this can be done best by first wrapping the child tightly into a blanket, arms included.

All cases—and at each treatment—are set up by a medical officer, and during treatment, if necessary, the head steadied either by himself, by a radiographer, or by a nurse—or, if available, one of the parents. The usual precautions

(wearing of lead-apron and gloves and keeping out of the direct beam) are observed. This duty is taken in turn by a rota of assistants, so as to minimize exposure still further. In older children it may be possible after a few treatments to leave the immediate neighbourhood of the treatment couch and direct operations from a corner of the treatment cubicle, and later still from outside it. The installation of a two-way microphone-loudspeaker system helps with some children, but its use may scare others, and the whole approach must be a rather individual one.

Dosage.—Daily treatment are given, the daily dose varying from 100 r. to 300 r. to one or two fields, and our course has lasted from 3 to 6 weeks. The total tumour dose has varied, and depended partly on the progress observed ophthalmologically. Still more varied has been the total skin dose applied to individual fields. The highest was 4750 r. in 57 days. This maximum dose was 50 per cent higher than the 3100 r. and 3200 r. given to 2 fields of another case; all other applied skin doses were below 2700 r., and most between 1000 and 2000 r. In many cases a moist desquamative skin reaction was produced, which healed within a month, leaving no lasting sequelae in the skin.

Dosage and result.—Successes have been obtained with deep X-rays by daily tumour doses of 80 r. as well as 200 r, and by treatments lasting 16 or 57 days, but the number of cases observed is too small to confirm the impression that neither dose rate nor the total time are of critical importance within a wide range. The total estimated tumour dose, however, showed a critical range: the successes were achieved by tumour doses of 3300 r. to 4400 r. irrespective of whether radium-moulds or deep X-rays were used, including one case in whom radium-moulds of increasing strengths were applied at short intervals within 5 months, the highest single dose being 3800 r.

Two main treatment schemes have been used: either a single "radical" course or repeated courses of deep X-rays, in each one of which a dose was given just sufficient to make the tumour disappear. This was usually achieved by doses of 2000–3000 r., to be repeated as soon as there was renewed activity. The latter scheme is somewhat similar to that recommended by Martin and Reese (1936, 1942, 1945), but in our hands it has never been successful, whereas the single radical course of treatment has given us outright cures.

We have only once had the opportunity of treating a unilateral case *ab initio*. This was a girl, aged 3 years and 9 months, who came with a history of over 2 years but still only a comparatively small growth. A total tumour dose of 2160 r. was given in 24 days and the lesion regressed. Ten months later, however, there was a large recurrence and the eye was excised. Histology confirmed the presence of active growth.

Sometimes it has been necessary to continue the first course up to a much higher tumour dose. There was one case in our series where even the second eye carried a growth covering almost the whole of the retina—he had come to us with simultaneous affection of both eyes. After the excision of the first eye, which was completely filled by growth, a tumour dose of 5730 r. was applied to the remaining eye. Within 60 days there was some regression, but definite activity was noticed again less than 4 months later and a further course of deep X-ray therapy started. This did not hold up the fatal issue and, in addition, there was recurrence on the enucleated side and massive secondary involvement of the liver (Case report 3).

In this part of the body as anywhere else—but perhaps even with greater force—is one struck by the confirmation of the hypothesis most prevalent for the explanation of failures through over-dosage. We know that in most of these cases the mechanism of healing is by calcification, a process presumably brought about by the connective-tissue stroma of the tumour bed—and occurring even spontaneously in a number of well authenticated cases (Hine, 1937). Over-dosage will damage the normal tissue—and the tumour bed—in such a way that it fails to produce the curative fibrosis and calcification; or even when once started, this process may break down at some future date, and allow the growth to re-establish itself from a small number of still active cells or cell nests.

Post-operative irradiation.

In 3 cases, in 2 of which there was optic nerve involvement, post-operative irradiation was given after enucleation of the eye.

A radium mould was used in two cases and deep X-rays in the other. Two fields are used in the deep X-ray technique, an anterior and a lateral orbital, the field sizes being usually somewhat larger than in the primary technique, i.e. 5 cm., and the focus skin distance greater (40 cm.).

"Tumour" doses varied from 2560 r. (D.X.R.) to 3570 r. (Ra-mould), but in one case only 660 r. were apparently given (by Ra-mould), and this furnished the only recurrence in this group (Case 19). We have not as yet given post-operative treatment to the orbit of the removed eye in bilateral cases. This may be advisable as a general policy in view of the ultimate fate of some of them, i.e. local recurrence even where no apparent extra-ocular extension was originally present, unless one regards these deposits as due to retrograde extension from the second eye, which by that time has got completely out of control. One should also remember, as Duke-Elder (1940) points out, citing Meighan and Michaelson (1938), that there may be discontinuous spread in the nerve, so that the excised specimen (even if part of the nerve is included) need not necessarily show the extra-ocular extension which has in fact already taken place. In carrying out this suggestion the contribution that the lateral fields will make to the retained retina or the enucleated orbit respectively and which will amount to a depth dose of about 20 per cent must be taken into account. This technique has been used in a recent case which had a recurrence in the excised orbit as well as growth in the second eye, and is also applicable in the "prophylactic" irradiation of a case in which bilateral enucleation has been performed.

When we first collected these cases it appeared as if the unilateral cases treated post-operatively by irradiation would not be of very great interest because surgery alone might have been responsible for the good results. We have, however, in our series one case of recurrence within 6/12 of the operation (this was treated by a combination of deep X-rays and implantation of radon-seeds, but recurred again and proved rapidly fatal), and the above-mentioned case, which may be regarded as having been inadequately treated in the post-operative period. This case had a remarkably long latent period of 5 years, and a very large dose of irradiation was necessary to arrest the growth. The question of post-operative prophylactic irradiation in this disease should therefore still be regarded as an open one.

Surgery.

Surgical excision is the mainstay of therapeutic procedure in this condition. All bilateral cases in this series have had at least one eye excised—in one case which was sent to us for radiotherapy of the second eye the growth was already too advanced and this eye, too, was removed. (The child is alive without any sign of recurrence 5 years later.)

The three primary unilateral cases treated by surgery alone are still alive and well $3\frac{3}{4}$, $5\frac{3}{4}$ and 6 years later.

The type of operation performed consisted of an excision of the globe with as much of the optic nerve as possible.

Recurrent cases.

There were 3 cases in our series of recurrence after radium or radon implants or diathermy to growths in the remaining eye. All these were treated by deep X-rays. Two cases had several courses which failed to arrest the disease, and they have died 18 and 28 months after the first course. The third case, having been given 3400 r. tumour dose, developed chronic iritis and cataract within 4 months and the eye had to be removed. No growth was found on histological examination.

One case of massive recurrence in the orbit with bone involvement after enucleation of the only affected eye failed to respond to combined irradiation by deep X-rays and implantation of radon needles; another case, successfully treated, is of considerable interest. It is the case already mentioned which received a post-operative prophylactic dose of 660 r. by a radium mould and developed a recurrence 5 years later. The case illustrates several points of general radiological interest, and is of special importance as regards the natural history of this disease (Case report 19).

Cataract.

Radiation cataract, the commonest complication of radiotherapy of the eye, developed in 5 cases (45 per cent). One, having received 3400 r. by deep X-rays in 16 days for a recurrence after treatment by radon-seeds elsewhere (tumour dose estimated at 2000 r.), was complicated by chronic iritis and needed extraction of this the remaining eye 4 months later (now $5\frac{1}{2}$ years ago)—no growth was found on histological examination, as already mentioned. The second case had 3300 r. also in 16 days, but did not develop cataract until 3 years later. This was needled recently. Another case developed its cataract $2\frac{1}{2}$ years after receiving 4400 r. (in 57 days); this was extracted 4 years ago and vision is only partial now. Another cataract that was removed was obvious already 9 months after a dose of 3000 r. (given in 34 days at the age of 7 months), and the eye has since been excised for a recurrence of the neoplasm. The last case, treated by a radium mould (the dose in the lens lying between 5300 r. on the surface and 3000 r. estimated for the retina), developed a small posterior polar cataract not interfering with vision, $2\frac{1}{2}$ years later. She received normal schooling, and is now—at the age of 15—able to read ordinary print, if with some effort.

The dose-time relationship together with the age of the child appear to determine the time of onset of the cataract. The 2 early cataracts (i.e. within less than 12 months of treatment) appeared in children irradiated at 7 and 16

months of age, whereas the comparatively "late" cataract was seen where treatment was given at the age of $2\frac{1}{2}$ and 4 years. One other case in whom the daily tumour dose was less than 80 r. (at the age of 15 months) also developed a "late" cataract.

Case reports.

Case 3.—M. B—, a boy, 4 years of age, was first seen at the Middlesex Hospital on 29.i.41 with a history that 7 months previously his mother had noticed a white spot in the right eye and 5 months later similarly in the left eye. His general condition was good, but he was rather drowsy. Rt. eye: whole eye filled with whitish neoplastic mass extending down from medial part of retina. Small portion of normal retina seen. Lt. eye: Perception of light and ?? counts fingers.

3.ii.41: Excision of right eye (Mr. M. H. Whiting). Histology: Glioma retinae of retinoblastic type. Optic nerve involved.

4.ii–2.iv.41: Course of deep X-rays (25 treatments) to left eye, using 4 fields (lat. 2700 r., ant. 1400 r., sup. 1800 r., inf. 1500 r.), each 3 cm., at 200 kv., 10 Ma., Thoraeus filter, $\frac{1}{2}$-val. layer 2 mm. Cu, F.S.D. 35 cm. Tumour dose 5727 r.

Meanwhile, on 17.ii.41 the child began to be brighter, and on 3.iii.41 the growth appeared smaller. 13.v.41: Lt. eye looks improved. Counts fingers. 8.vii.41: Sight better. O.E.: Shallow detachment temporal side and some shrinking of growth on nasal side. 1.viii.41: Seeing less well, but distinguishes light and dark. O.E.: Most of retina shows dead white.

2–15.viii.41: Further course of D.X.R. to 3 fields (same technical factors). Tumour dose 2785 r.

9.xii.41: Readmitted to Sector Hospital. Very pale, unhappy, miserable. Vision: Can see light and crude shapes. No light reflex. Three weeks later he developed an enlarged liver and severe anaemia (Hb 25 per cent., R.B.C. 1·7 m.) due to rupture of the liver and resultant haemoperitoneum. Haemoperitoneum has not to my knowledge previously been reported in the literature as a complication of hepatic metastases due to retinoblastoma.

4.i.42: Died.

P.M. findings: *Skull.*—Many extradural haematomata (secondaries?). *R. orbit.*—(Eye excised 11/12 previously). Bone eroded into sella turcica. Right greater wing of sphenoid dark red and friable; the red friable mass extends as far as the left orbit but not into it. *L. eye.*—(Treated by D.X.R. 5 and 11 months previously.) Some of the muscles of the eye red and haemorrhagic. *Abdomen.*—As above. *Histology.*—Diffuse infiltration of the left eye, bones, and liver with retinoblastoma (i.e. similar to the original histology).

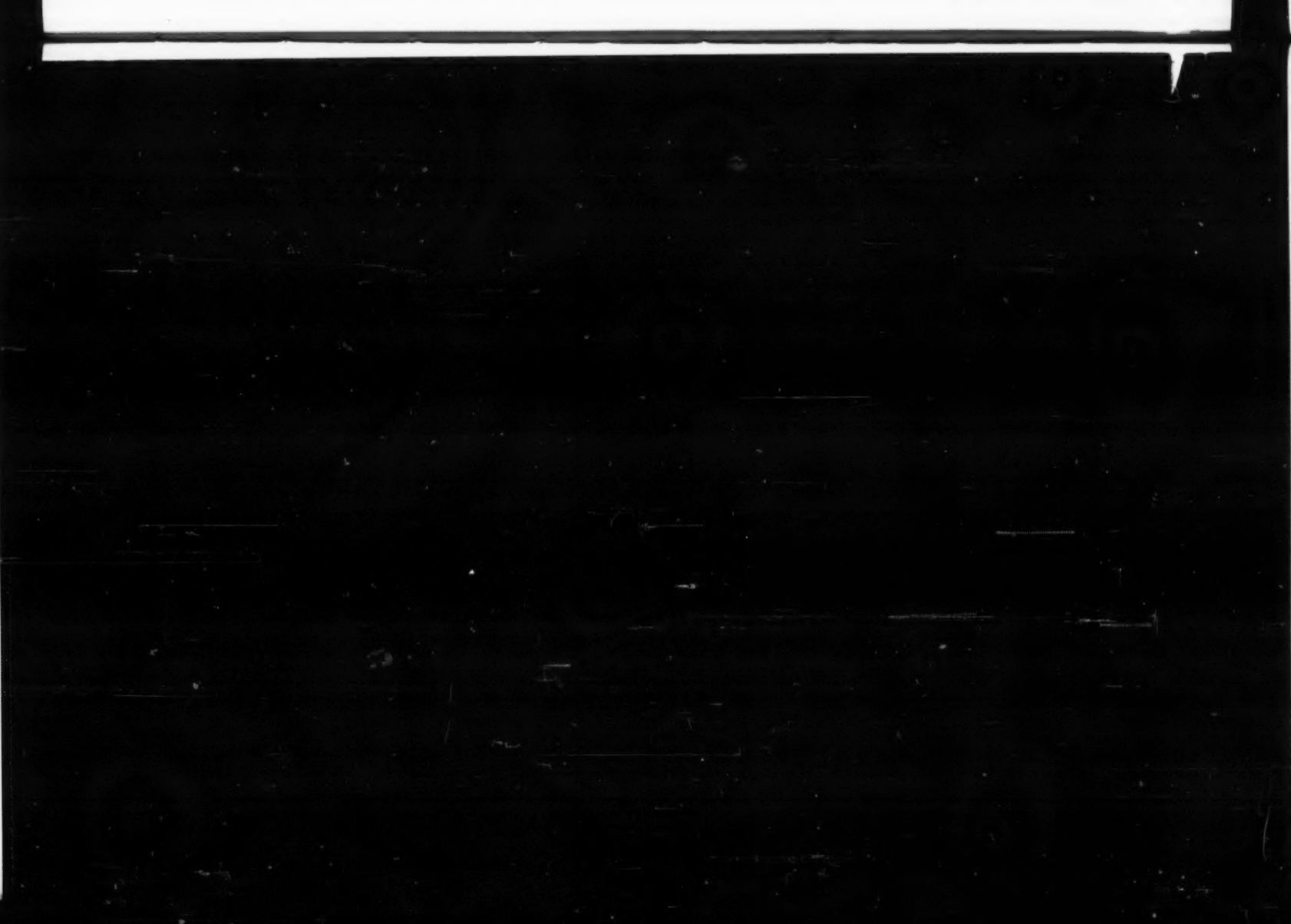
Case 14.—J. L. H— first came under observation at the Middlesex Hospital in July, 1945, at the age of 9/12, having had his left eye excised elsewhere 3 months before (histology showed retinoblastoma without involvement of the optic nerve), and at the same time a focus the size of a disc in the periphery of the right retina had been isolated by diathermy barrage. At this time there was no recurrence in the socket, but two new foci of growth had appeared in the right eye. He was given a course of deep X-rays (T.D. 1750 r.), and when in February, 1946, there was again obvious activity a further 3000 r. was given.

1.

2.

FIG. 1 and 2.—Case 19, D. M—, 17.x.46. Showing calcified (healed) glioma retinae in right orbit.

Japha.



Eight months later the old foci looked less active and more solid. About a fortnight after this examination the boy began to vomit (apparently in a rather projectile manner) and became drowsy. When examined again on 20.xi.46 there were signs of brain-stem involvement, and a course of deep X-rays directed at this region was started. There was some temporary improvement, but the child died within 3 months of the onset of the final stage. Lumbar puncture during this time showed the presence of peculiar mononuclear cells in the cerebrospinal fluid, which in the light of the later post-mortem findings must be regarded as retinoblasts. A section of the midbrain revealed a column of growth in the aqueduct. Besides this there was, on histological examination, active growth in the right eye with involvement of the optic nerve on this side as well as in the stump of the left optic nerve, and in the pia arachnoid over the cerebral hemispheres and midbrain. Multiple small secondary nodes were present in the liver.

Case 19.—D. M—, a boy, 5 years of age, was sent to the Middlesex Hospital in 1929 from Jersey, where 5 months previously his right eye had been removed for glioma retinae. A radium mould was applied as a "delayed post-operative measure," but the surface dose when recalculated recently was found to have been only 660 r. (in 36 hours). Nearly 5 years later there was a large swelling in the orbit which was treated by X-rays in Jersey (details unknown) and the swelling disappeared. A few months later a further course of X-rays was given for a "small hard bean-shaped swelling" in the lower half of the orbit.

Seven months after this the mass was again thought to be larger and the boy referred back to the Middlesex Hospital in September, 1936. He was found to have "a hard mass 2 cm. in diameter protruding from the inferior wall into the orbit." Treatment was given by a nidrose and elastoplast radium applicator (radium skin distance 3 cm., area 56 cm.^{2}, charge $2 \times 6{\cdot}6 + 3 \times 10 + 2 \times 13{\cdot}3 = 69{\cdot}8$ mg., filtration 1 mm. Pt., time 283 hours) and in 2 weeks the eyelids received a dose of 11,000 r., the estimated dose at the posterior orbital wall being 6000 r. The boy was lost sight of during the war, but reported again as a young man in October, 1946, that is, 10 years later, when he presented with marked telangiectases on the right cheek around the orbit and a small sinus over the zygomatic arch. A skiagram showed a certain amount of bone necrosis in the frontal and malar bones and also an irregular calcified mass in the orbit (Fig. 1 and 2). The other eye has remained normal.

SUMMARY.

The literature on the treatment of glioma retinae is reviewed, and 19 cases seen at the Middlesex Hospital between 1929 and 1946 are described as regards age and sex incidence, length of history, side affected, family history, pathology, including some remarkable post-mortem findings, and their treatment discussed in detail. The results of radiotherapy are summarized in Table VII.

(a) *Post-operative irradiation* has been successful in the two cases where adequate treatment was given irrespective of whether a radium mould or deep X-rays were applied. In the one case described at length above, the first treatment, where 660 r. only were given, must be regarded as quite inadequate, but the recurrence which developed 5 years later responded well to a radical course.

(b) *Primary irradiation.*—The two cases treated by radium moulds and two of the three cases treated by deep X-rays more than 5 years ago are alive and

well 15, 10, 5 and 5 years after treatment. Cataract has developed in three of these cases and two have had operations for its removal. The sight in these three cases is partial only; the fourth case has normal sight. A minimum tumour dose of 3300 r. is considered essential, and overdosage (which may lie at anything more than 4500 r.) is warned against.

I wish to express my grateful thanks to Mr. Greeves, Mr. Whiting and Mr. Goldsmith, under whose care many of these cases were admitted, and whose ophthalmological examinations have formed the basis upon which the radiotherapist has been able to build. My special gratitude belongs to Professor Windeyer, who directed the treatment of all the radiotherapeutic cases, for his unfailing encouragement and many valuable suggestions.

TABLE VII.—*Results of Radiotherapy* (15 *Cases*).

	Primary Cases (11). Treated.			Recurrent Cases (4). Treated.			Total.	
	More than 5 years ago.		Less than 4 years ago.	More than 5 years ago.		Less than 4 years ago.		
	Alive and well.	Died.	Alive and well.	Alive and well.	Died.	Died.	Alive and well.	Died.
Bilateral	4	1	2(*a*)	1(*b*)	1	1	7	3
Unilateral	3*	—	1(*a*)	—	1	—	4	1
Total	7	1	3	1	2	1	11	4

(*a*) (*b*) Eye excised following failure of radiotherapy for (*a*) recurrence, (*b*) chronic iritis and cataract.

* Post-operative irradiation. One case lost sight of after 33 months. One case treated for recurrence 5 years later—now 12 years ago.

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THE STUDY OF CONNECTIVE-TISSUE REACTION TO RADIATION. THE SIEVE OR CHESS METHOD.

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THE focusing of attention on the malignant cell in the study of tumour tissue reactions to radiations and growth inhibitory substances has not brought us nearer the state of affairs where both practical and theoretical issues of oncology and treatment of tumours can be studied with a better understanding and fairer prospects of success.

The importance of the stroma reaction to tumour and stromal reaction to radiation has been accepted for a long time, but only with generalizations on changes in " blood supply " and " tumour bed," leaving the whole problem of connective tissue reaction in a hazy state.

As regards tumour tissue reaction to radiation, much stress has been laid in the past on the changes following irradiation in the cells alone, but very little attention has been paid to the intercellular tissue components. Although recent advances in cytology and genetics have enhanced the importance of the cellular nucleus, it must not be forgotten that evidence, both experimental and clinical, is available to show that the effect of ionizing radiations on cells alone " is not the whole story of the events leading to the destruction of proliferating tissues and that other factors come into play " (Muller, 1940).

The great importance of connective-tissue reactions to X-rays in radiotherapeutics has been raised by Windeyer (1942), Ellis (1942), Windholz (1947). The preservation of the connective-tissue structures which contribute to repair and recovery has been taken into account in a quantitative biological control of optimal and effective dose levels in X-ray and interstitial radium therapy, and the importance of the peripheral neighbouring regions which exercise an influence on the reaction in the irradiated areas or volumes of tissues has been stressed (Jolles, 1946*a*, *b* ; 1947 ; 1948).

Conceptions of indirect action and effects on tumour cells are coming more and more to the forefront, and a clear definition of aims and avoidance of terms borrowed from radiochemistry seems of great importance.

In the study of the tissue reactions to ionizing radiations several effects have to be gone into separately :

(1) The direct effect of radiations on cells (intracellular effect (Koller and Smithers, 1946)).

(2) The direct effect on the intercellular elements.

(3) The effects on cells caused by the stroma (stromal effect).

(4) The effects on cells and stroma in the irradiated tissues caused by the neighbouring tissues (external effect, Jolles).

It is obvious that only by means of special methods and technical arrangements can these effects be studied.

A novel technique which has been devised in order to overcome some of the difficulties is described.

MATERIAL AND METHOD.

Material for histological examination can be obtained from accessible tumours (breast, skin) irradiated through a lead sieve with circular or square apertures of diameters varying from 1·0 to 2·0 cm. Very advanced, inoperable tumours of breast in which only some palliative treatment can be given, or tumours of skin which, because of their extent, situation, infiltration of surrounding and underlying structures are not amenable to either radical surgery or radiotherapy, were used in this investigation.

The difficulties arising from the normal variability of a breast tumour in different areas of the same tumour have to be kept in mind. The cellular carcinoma simplex with minimal fibrosis is the most suitable, and if this structure is found in the first pre-treatment biopsy, it is highly probable that suitable fields for comparison will be found in sections from subsequent biopsies. In examining the sections an endeavour must be made as far as possible to compare areas at the same depth from the surface, and with a similar arrangement of tumour cells. In ulcerated tumours inflammatory changes, especially oedema, make the critical interpretation more difficult and therefore the sections have to be made in selected points. Consecutive sections are taken from not too distant parts of the tumour.

The lead sieve is applied tightly over the part to be treated, and the periphery of the lead mask is marked on the skin, as are also marked the centres, of the apertures. This is most important in order to secure landmarks for appropriate exposure of parts which have to be irradiated, and to shield the areas to be screened off the beam during treatments taking place on consecutive days.

Tattooing of the skin has to be ruled out because of the complicating foreign body reaction. Mapping out of the " chess " squares necessary for the irradiation

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EXPLANATION OF PLATES.

FIG. 1.—Stent mould applicator fitting tightly, with recess for " chess " lead panel and X-ray tubehead applicator.

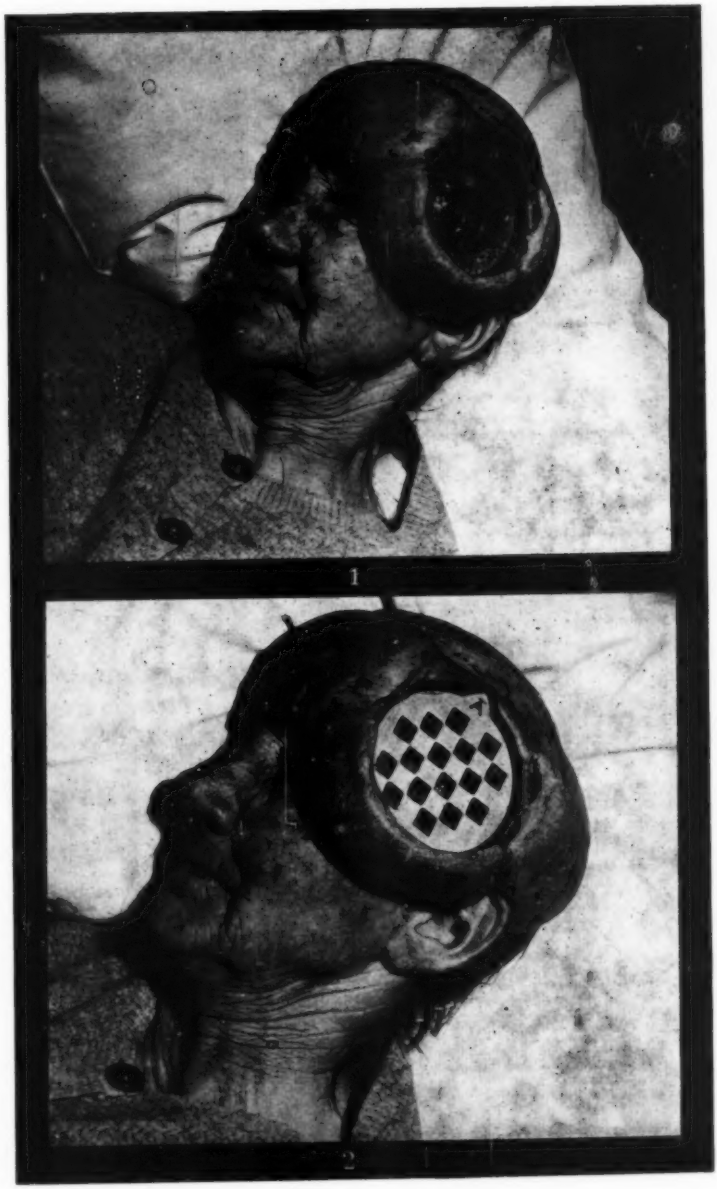
FIG. 2.—" Chess " lead panel in position.

FIG. 3.—*Case* 3. Exposed area. Section taken after 5400r were given through " sieve " in 14 treatments spread over 20 days. The " hypertrophy " of connective tissue is very well marked ; it consists of the deposition of an excessive amount of collagenous fibres, which is induced by the radiation. The tumour cords embedded in this grossly altered connective fibrous tissue exhibit uniform degenerative changes. They show excessive nuclear pyknosis and cytoplasmic vacuclization—both can be attributed to diminished or completely suppressed food supply. Cellular injuries in tumour cords which could be referred to direct radiation effect on chromosome mechanism are very rare.

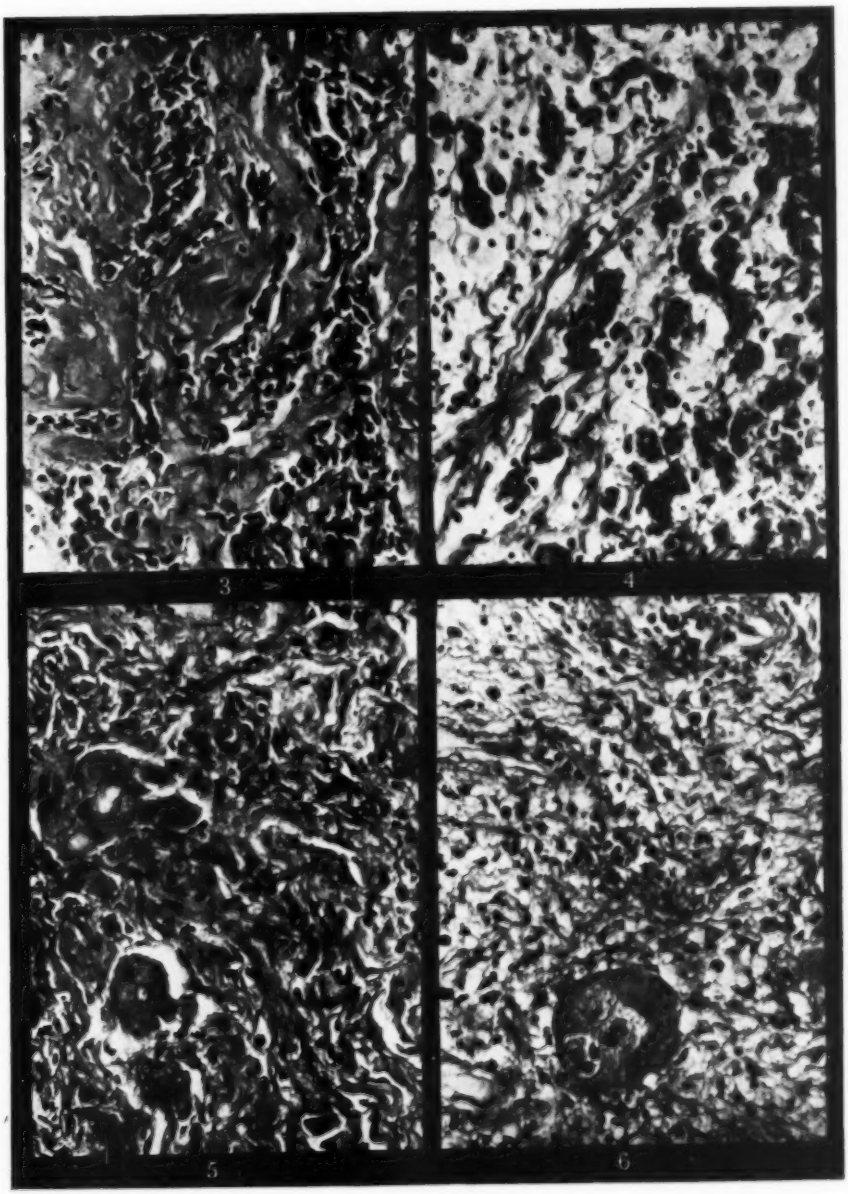
FIG. 4.—*Case* 3. Protected. Tumour tissue represented by thin infiltrating cords which are embedded in loose connective tissue. The latter contains a very small amount of extracellular matrix, and it can be seen that the collagenous bundles are similarly very scanty.

FIG. 5.—*Case* 4. Exposed. Section taken after tumour received through " chess." 4000r in 14 days and 2500r on each glancing field also in 14 days. Fibrous hypertrophy of connective tissue is very marked ; cells in tumour cords show cytoplasmic vacuoles and deeply stained pyknotic nuclei.

FIG 6.—*Case* 4. Protected. Tumour cords infiltrating connective tissue. The latter is invaded from the capillaries by polymorphonuclear leucocytes and lymphocytes.



Jolles.



Jolles.

technique does not help in the localization of the borders between the irradiated and protected sectors on the histological slide. This presents special difficulties when the dose of radiation delivered is small, or when one studies in serial sections the very early stroma reactions.

In tumours of the skin, always, and in breast tumours whenever possible, a special mould applicator is built for each case (Fig. 1 and 2). The lesion is reproduced by means of a stent impression in a plaster cast, over which a tightly fitting stent or black-tray compound applicator is built in such a way as to be easily mounted over the part of the body, and fitting in such a way as not to allow even minimal shifts of the applicator, and so avoid the X-ray beam striking the alternate " opaque " squares of the lead chess which is recessed in the mould.

The radiation has to be delivered at right angles with the X-ray tube head applicator directed vertically. In order to avoid oblique penetration into areas beneath the opaque squares the lead chess must be in direct touch with the tumour surface. It is found in the course of treatment that owing to shrinkage of the tumour the recess sleeve in the stent mould into which the " chess " is slipped has to be filed down from time to time during the course of treatment.

The thickness of the lead, which is limited for practical considerations, permits 3 per cent of the radiation to pass through on to the protected areas. To that amount of radiation received by the not directly irradiated areas has to be added about 3–5 per cent of scatter radiation from the neighbouring exposed areas. This varies according to the size of the apertures, and decreases towards the centre of the " opaque', squares. The size of the squares has been chosen of not less than 1 cm., so as to leave an adequate amount of tissue either almost completely non-irradiated or exposed for biopsy taking.

Sections are taken before treatment is commenced and then at varying intervals. Cuts are made into the exposed and protected sectors, and also one section is made cutting across both the exposed and protected areas of tissue.

Accurate labelling of biopsy material must be insured, as no other way of recognizing the protected and exposed sectors on the histological slide is available. A working chart is found indispensable. Prior to taking the biopsy the lead mask is applied and the exposed areas are mapped out by means of gentian violet paint.

The biopsy material was fixed in sublimate-formol solution or Sousa's solution. Following paraffin embedding, sections were cut at 7μ and stained with (1) Harris' haematoxylin, eosin, (2) Van Gieson stain, (3) by Gallego's method, and (4) silver impregnation.

The irradiation was taking place every day, 5–6 days per week. The setting up was performed always by the same people, and every precaution taken in order to avoid minimal shifts.

PRELIMINARY RESULTS.

Nine cases have been dealt with so far. Cases suitable for this investigation are not frequently encountered, especially as they have to be kept in hospital for 6–10 weeks. The critical interpretation of the slides is very difficult. Comparable areas in the protected and exposed parts have to be studied only. Two cases are described in detail:

Case 3.—Mrs. Emma M—, aged 67. Cancer of breast, right. Stage III. Cellular carcinoma simplex.

15.x.46 : Two years' history. General condition poor. Sprouting ulcerated growth in right breast, upper outer quadrant, fixed to pectoralis. Ulcerated area 7·5 × 6·5 × 2·5 cm., with several skin noduli scattered over breast area. Hard mass 4 × 3·5 × 3 cm. in axillary tail and hard glands in right axilla.

15.x.46 : Treatment commenced. 140 kV., 5 mA., 25 cm. F.S.D. 0·25 Cu + 1 Al. filter applicator 12 × 10 cm. Treatment through sieve, 1 cm. diam. circles. 17 treatments in 21 days. 6000r given.

16.x.46 : X-ray chest : No evidence of secondary deposits.

7.xi.46 : Treatment concluded.

Serial sections taken : (1) At commencement of treatment. (2) After 14 days ; 3800r given. (3) Three weeks after commencement of treatment; 5400 r given. (4) Section taken 7 weeks after commencement of treatment, e.g. 4 weeks after conclusion of treatment (6000r in 21 days).

No hormonal treatment was given till the middle of 1947. This was not well tolerated. Subsequently the patient had a pathological fracture in the left femur and died early in 1948 (Fig. 3, 4).

Case 4.—Mrs. Margaret A—, aged 64. Cancer of breast, right. Stage III. Carcinoma simplex.

12.xi.46 : Six months' history. General condition fair. Hard mass filling right breast, fixed to pectoralis and ulcerating skin in anterior axillary line over an area 8 × 5 cm. with a sloughy crater and rolled-up edges. Hard glands in right axilla. Admitted to ward. Sulphathiazole dressing ; applicator built, etc. X-ray chest : No evidence of secondary deposits.

4.xii.46 : Treatment commenced. Chess squares 2 × 2 cm., 180 kV., 10 mA. 0·5 Cu. filter, 40 cm. F.S.D. 40 r/min. Applicator 15 × 10 cm.

Sections taken : (1) Before commencement of treatment. ; (2) After 4 days ; 1500r given in 4 treatment. (3) After 14 days from commencement of treatment ; 4000r given in 14 days in 12 treatments. At this point " chess " technique abandoned and routine glancing technique reverted to. (4) Section taken when tumour received apart from the 4000r with the chess technique 2500r on each glancing field in 14 days ; 10 treatments. (5) Section taken at end of treatment (4000r to chess and 3500r on each glancing field in 5 weeks). She is remarkably well to date. When last seen, 15.xi.48, there was no sign of activity, and a small 2 sq. cm. area of granulation was healing up (Fig. 5, 6).

GENERAL CONCLUSIONS AND INFERENCE.

From the material already available a few data can be submitted.

(1) The " sieve-chess " technique is a workable one for the study of direct and indirect effects of irradiation.

(2) The connective-tissue reaction takes place not only in the irradiated areas, but also in the neighbourhood protected from the direct irradiation.

(3) There are visible effects on cells not directly exposed to the radiation.

(4) The pattern of connective-tissue destruction and regeneration can be studied.

As a general inference it can be said that the irradiation affects the connective tissue cells and induces a kind of " over differentiation." This consists chiefly in the deposition of an excessive amount of collagenous fibrils, which are organized into solid, coarse bundles in the exposed parts where the fine connective-tissue

network is grossly altered. Lymphatic channels and blood capillaries are greatly affected, compressed and blocked. This has a considerable effect on the tumour nourishment, the lack of which enhances tumour tissue degeneration.

SUMMARY.

A new method for the study of connective-tissue reaction in tumours to radiation is described, and a few preliminary data of the investigation carried out so far are given.

I am greatly indebted to Prof. R. W. Scarff, The Middlesex Hospital, for his interest in this investigation.

My thanks are due also to Dr. P. C. Koller (Chester-Beatty Institute, Royal Cancer Hospital (Free), and Dr. W. Bryan (Morbid Anatomist in this hospital), for their study of the slides and most helpful criticism. The help of Mr. E. J. Pamely (Research Technician of this Department) and Mr. C. Clarke (of the Pathological Department) is also acknowledged.

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FURTHER OBSERVATIONS ON THE PROTECTION EFFECT IN RADIATION CHEMISTRY.

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IN a series of papers (Dale, 1940; Dale, Meredith and Tweedie, 1943) it has been shown that the action of X-rays on aqueous solutions of biologically active compounds was indirect, i.e. through some change of the solvent (water), which in turn acts on the solute. Further, it has been shown that in consequence of this indirect action, if two solutes are present, the second solute (the protector) reduces the radiation effect on the first one (the indicator) (Dale, 1942; 1943), by sharing the intermediate product formed from water by the radiation. The indicator, therefore, appears to be "protected," and this protection tends to be specific (Dale, 1947). A further refined analysis of this protection phenom-

enon revealed certain unexpected quantitative relationships between the protective power of a substance and its concentration, which will be described and discussed in this paper, together with the closely linked ionic yield of the indicators used, the knowledge of which is the necessary basis for the quantitative treatment of the experimental results. The fact that these new results were derived from experiments with two indicators of very different molecular weight, for an extended range of protective substances added to both indicators, helps to build up a more complete picture than has been obtained hitherto. The experiments will be described under the following three headings :

(1) The variation of ionic yield with concentration for carboxypeptidase solutions (C.P.), and alloxazin-adenine-dinucleotide solutions (D.N.).

(2) The specific protective action of unit mass of various solutes when irradiated (*a*) in presence of C.P. and (*b*) in presence of D.N.

(3) The variation of the protective power per unit mass of protector when the concentrations of protector and indicator are varied (phenomenon of the " changing quotient ").

Irradiation Technique.

The majority of the experiments were carried out with radiation of half value layer 5·2 mm. Cu (average $\lambda = 55$xu) from a continuously evacuated 500 kV tube, and a few with radiation of 1·6 mm. Cu H.V.L. (average $\lambda =$ 130xu) from a 250 kV tube. Whenever possible, the solutions, contained in Pyrex glass tubes, were spaced at such distances from the target that each received the desired dose during the same overall treatment time, care being taken to avoid shielding of one tube by another. This ensures that the relative doses received by different tubes are not affected by unavoidable fluctuations in the tube output. The absolute dose delivered was monitored by a Hammer integrating dosemeter which had been calibrated against a substandard meter.

Chemical Technique.

(a) *Carboxypeptidase.*—The crystalline enzyme was prepared, and its activity measured by the method described by Anson (1936–37), using chloracetyl tyrosine as the substrate. The stock solution of enzyme and the solutions of protective substances were made up with water, glass re-distilled from alkaline potassium permanganate, and the pH adjusted so that a sample reacted pink to phenolphthalein. All necessary dilutions were carried out with glass re-distilled water adjusted to the same pH by the addition of 1 ml. of 0·02N NaOH to each 100 ml. of water. The enzyme solution required for any experiment was made up as late as possible before the start of the experiment. Experiments with very high concentrations of C.P. were performed during this series of experiments, and some special difficulties were encountered. These arise because very high doses of radiation (one to two million roentgens) are required to achieve a reasonable degree of inactivation in solutions with concentrations of the order of 10 per cent or more. There is a strong tendency of re-crystallization during the long exposure to radiation, which makes it almost impossible to keep the enzyme in solution at a pH compatible with its stability. However, it was found that a 6 per cent solution could be maintained for most of the time by stepwise addition of NaOH. This procedure is less accurate than that which

was used for the highest concentration, i.e. 15 per cent. In this case a suspension of crystals of known activity was centrifuged in capillaries of uniform inside diameter of between 2·5 and 5 mm. closed at one end, until a solid column formed. The concentration reached by this method was derived from the known concentration of the suspension by measuring the height of the column of crystals after, and the height of the suspension before centrifugation. The supernatant fluid was then removed, the capillaries closed by fusing, and inserted into a glass tube through which tap water could be passed to supply constant cooling during the long irradiation time. After irradiation, the top of the capillary was cut off above the level of the solid column, and the part containing the enzyme crushed in a beaker containing just sufficient N/10 NaOH to dissolve the enzyme, and then diluted to an appropriate concentration for the activity test.

(b) *Dinucleotide.*—The specific protein and the barium salt of alloxazine adenine dinucleotide (D.N.) were prepared according to Warburg and Christian's method (1938). No adjustment of pH prior to irradiation is necessary. The oxygen uptake ensuing when the complete amino acid oxidase acts on the substrate alanine is only an approximate measure of the amount of D.N. present, since the mass of D.N. is not directly proportional to the oxygen uptake. To obtain the absolute amounts of D.N., a conversion of the oxygen uptake into amounts of D.N. has to be carried out according to the dissociation formula of the protein—D.N. compound (Warburg and Christian, 1938). Since various preparations of the specific protein of the amino acid oxidase differ in their activity, and even any one preparation gradually loses activity when kept for some weeks, the oxygen uptake with a known amount of D.N. in each experiment was measured, and from it the oxygen uptake caused by an infinite amount of D.N. calculated. This figure is then used to find the amount of D.N. remaining unchanged after irradiation.

Experiments on ionic yield.

It was shown in early experiments (Dale, 1940) that the amount of indicator affected by radiation (in the case of C.P. or D.N., this being the loss of activity) was constant over quite wide ranges of concentrations. This implies that the ionic yield, which may be defined as the number of molecules changed (M) divided by the number of ions formed (N), is independent of concentration provided there are no complications such as back reactions (this will be discussed in greater detail later). The range of concentrations used for C.P. was greatly extended in the present work, going up to a 15 per cent solution, and the constancy of ionic yield was verified for solutions above 0·03 per cent. For weaker solutions the ionic yield falls, as is shown in Fig. 1. These results may also be presented, as shown in a previous paper (Dale, Gray and Meredith, 1949), by the formula:

$$D_E = 5{\cdot}2 \times 10^7 \,.\, C + 8{\cdot}9 \times 10^2 \quad . \quad . \quad . \quad (1)$$

where D_E is the dose in roentgens to inactivate the C.P. solution to 37 per cent of its initial activity, and C is the initial concentration in g. per ml.

Similar experiments were performed with various concentrations of D.N., and similar results obtained as shown in Fig. 2. It should be noted that the

ionic yield for D.N. calculated by Lea (1946) from earlier experiments by one of us is much lower than the value obtained in the horizontal part of the curve, and this is due to the fact that the concentration then used was of the order of

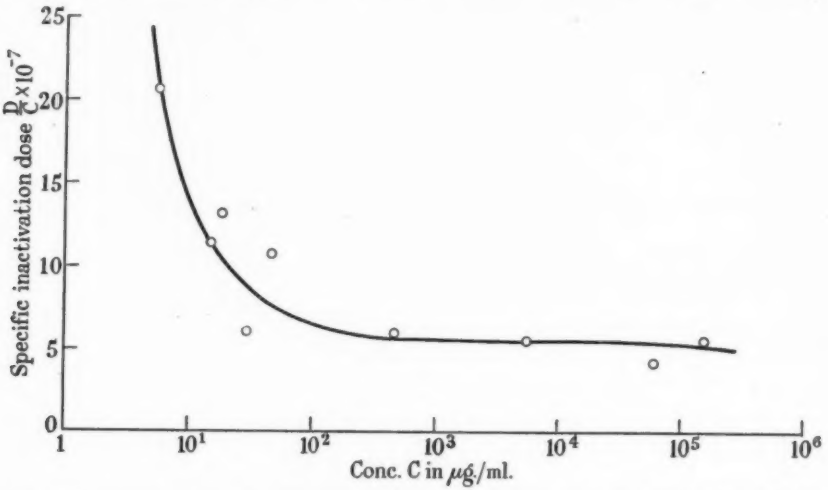

FIG. 1.—Relation between the reciprocal of the ionic yield and concentration for carboxypeptidase (C.P.).

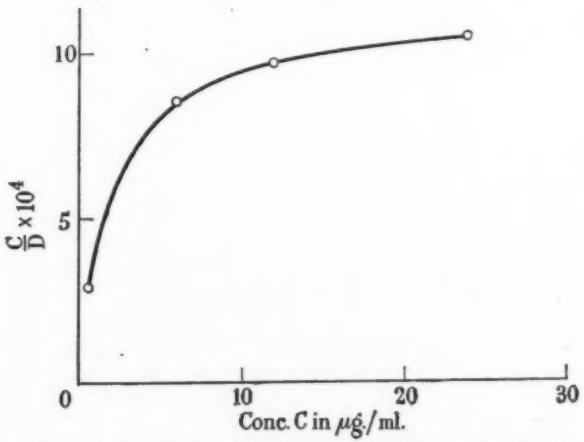

FIG. 2.—Relation between ionic yield and concentration for dinucleotide (D.N.).

0·6 μg. per ml., which is in the region where lower yields are obtained. The results for D.N. may also be expressed by an equation of the same form as that above, and we have in this case—

$$D_E = 9{\cdot}15 \times 10^8 \,.\, C + 1{\cdot}59 \times 10^3 \quad . \quad . \quad . \quad (2)$$

Protection experiments.

All these experiments consisted of measuring the interference with the radiation effect on the indicator substance by different concentrations of a variety of substances added to the indicator solution before irradiation. For the evaluation of the protective action, two irradiated solutions and two control solutions as follows were used :

(1) Irradiated sample of indicator and protector.
(2) „ „ „ alone.
(3) Unirradiated „ „ and protector.
(4) „ „ „ alone.

Solutions (3) and (4) compensate for any interference which can be caused by the sometimes high concentration of the protector. Such slight interference was actually only observed in the preliminary series of experiments with the very low concentration (10μg./ml.) of C.P. and there only in the case of albumin and alloxan.

In order to be able to compare the protective power of different substances, it has been expressed as the following quotient :

$$Q = \frac{D_{P+E} - D_E}{D_E} \times \frac{A_O}{q},$$

where D_{P+E} and D_E are respectively the doses, in roentgens, required to reduce the indicator activity to 37 per cent of its original value in the presence and absence of protector, and q and A_O are the weights of protector and indicator respectively per ml.

The quotient therefore expresses the ratio of the dose taken up by the protector to that taken by the indicator for equal weights of each.

The values of D_{P+E} and D_E were calculated from the degree of inactivation produced by a known radiation dose, by applying the experimentally established relationship between dose and effect (Dale *et al.*, 1943) :

$$\frac{A}{A_O} = e^{-kD/A_O}.$$

Where A and A_O are the final and initial indicator activities, D is the dose delivered and k a constant. For the sake of accuracy the degree of inactivation should be kept between the limits of 25–75 per cent.

In a previous report (Dale, 1947) the value of Q was found from the protection afforded by generally 100 μg. per ml. of a number of substances, added one at a time, to 10 μg. per ml. of C.P. Q was roughly constant, and it was concluded that the protective power per molecule is proportional to the molecular weight—perhaps not a surprising result, since all these substances have roughly the same average composition.

It is only when low molecular weight substances of special chemical configuration are examined that remarkable differences are found. Oxalic acid, which was earlier (Dale, 1942) found to be a poor protector of D.N., also proved to be a poor protector in the case of C.P., but if the C—C bond in oxalic acid is broken and the free valency of the carboxylic group linked to an H atom, thus forming formic acid, the protective power is raised about 200 times, and similarly, if

the O atom in urea is substituted by a sulphur atom, thus forming thiourea from urea, a change from a very low protective power to one about 1000 times greater occurs. If, however, the oxalic acid is modified by the addition of a carbonyl group (mesoxalic acid), or if urea is coupled to mesoxalic acid (alloxan), then the low protective power is only slightly raised.

Some of these experiments were repeated in a number of cases using 30 μg. of C.P. per ml., which increases the accuracy of the experiments, and some further substances added to the list of protectors (Table I).

TABLE I.—*Values of* Q *for* 30 μg./ml. *of C.P., and for* 100 μg./ml. *of Protector.*

Protector.	Q.
Glucose	0·67
Urea	0·03
Dimethylurea	0·35
Thiourea	30·0
Dimethylthiourea	8·8
Egg albumin	0·09

Dimethylthiourea and dimethylurea were examined in order to see the effect of introducing two methyl groups. Dimethylthiourea shows a smaller protective effect than thiourea, though still appreciable, whereas dimethylurea is of the order of glucose.

In order to find out more about the chemical reaction of the high protective power of thio-compounds, a solution of thiourea was exposed to a high dose of radiation, which caused a precipitation of elemental sulphur.

The part played by sulphur and sulphur compounds was therefore further investigated by irradiating colloidal suspensions of sulphur or solutions of sodium thiosulphate with C.P. as indicator.

The colloidal sulphur suspension used in these experiments was prepared by adding 2 ml. of N HCl to 10 ml. of 0·1N $Na_2S_2O_3$. Sulphur is thus liberated according to the reaction :—

$$Na_2S_2O_3 + 2\ HCl = 2\ NaCl + H_2SO_3 + S.$$

The suspension of colloidal sulphur was then diluted to 100 ml., and 1 ml. of this solution made up with an appropriate amount of NaOH to pH 8·7 and a volume of 100 ml., and evacuated for removal of SO_2. This solution is so dilute (1·6 μg. of sulphur/ml.) that it appears perfectly transparent.

TABLE II.

	μg. P.	μg. S.	Q_P.	Q_S.
Thiourea	5	2·1	55	130
,,	1	0·42	69	166
Dimethylthiourea	5	1·54	18	58
Colloidal sulphur	0·4	0·4	91	91
,, ,,	1·6	1·6	110	110
$Na_2S_2O_3$	5·2	1·06 (one S)	24	118
		2·12 (two S)	24	59

Table II shows the results of these experiments, in which the absolute amounts of the protector (μg. *P*) are given together with the amounts of sulphur (μg. *S*) contained in the compound and the corresponding quotient (Q_P and Q_S).

On inspection of the quotient for comparable amounts of protector substances one has to conclude that—

(1) All these *S*-compounds have a very high protective power.

(2) The non-sulphur residue of the molecule plays some part, since the protective power of thiourea is not equalled by any of the other combinations with sulphur—colloidal sulphur coming nearest to thiourea. In the case of $Na_2S_2O_3$ one can be in doubt as to what extent the two sulphur atoms contribute to the protection, the two extremes being that both react equally or one only.

The "changing quotient."

Although the earlier results are qualitatively borne out by the new series of experiments, a new phenomenon was observed.

On varying the concentration of the protector it was noticed that in certain cases the value of *Q* was not constant, contrary to the assumption of a simple sharing mechanism of the product formed from water by irradiation.

Q declined with C.P. as indicator in the case of glucose, thiourea, sodium formate, dimethylurea and dimethylthiourea, as the concentration of these protectors increased, indicating that the protective power was progressively decreasing. This we have called the phenomenon of the "Changing Quotient," and Fig. 3 shows two examples of it. We have not observed the phenomenon with D.N. as indicator.

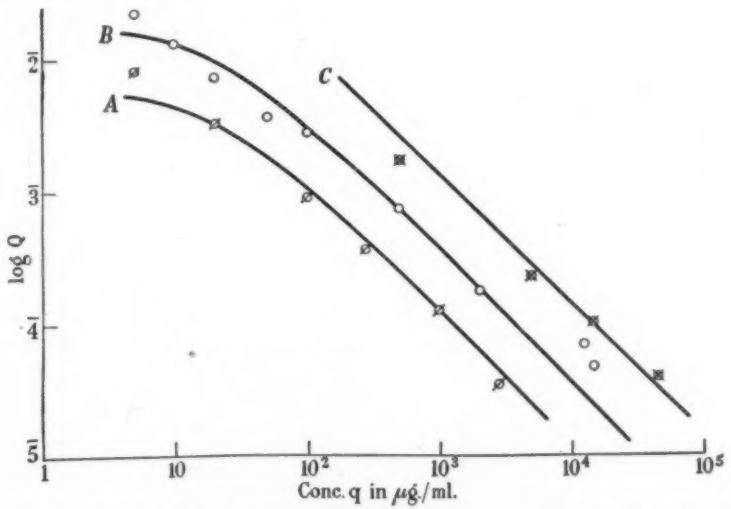

FIG. 3.—Curves showing "changing quotient" for carboxypeptidase (C.P.). Curves are theoretical, points experimental.

A. ø. Dimethylurea with C.P. (30 μg./ml.).
B. o. Glucose with C.P. (30 μg./ml.).
C. ×. Glucose with C.P. (90 μg./ml.).

DISCUSSION.

In an attempt to explain the indirect action of ionizing radiations on aqueous solutions, Weiss (1944) has suggested that it is due to the production of hydrogen atoms and hydroxyl radicals from the water by the radiation. We will adopt the concept since it is plausible and convenient for dealing with the problem in terms of kinetics. Our arguments, however, are not dependent upon its correctness, nor would they be invalidated by its disproof.

Lea, Smith, Holmes and Markham (1944) pointed out that, in addition to reacting with solute molecules, radicals may also recombine with one another, having a mean life, as free radicals, of τ secs. Thus the equation originally proposed by Dale *et al.* (1943) relating, for the case of a single solute (the indicator), the amount A of indicator unchanged after a dose of D roentgens to the amount A_O initially present must be modified to the form

$$A/A_O = e^{-\frac{Z_I p_R p_I}{Z_I p_R A_O + \frac{1}{\tau}} \cdot kD} \quad . \quad . \quad . \quad . \quad (3)$$

Where Z_I is the calculated number of collisions per second undergone by a single radical with the indicator molecules (Lea, 1946, p. 53) present in a concentration of 1 g.-molecule per litre, p_R is the probability that a radical will be destroyed in a collision with an indicator molecule, whilst p_I is the probability that such an event will also lead to a measurable change in the indicator molecule (e.g. inactivation of C.P. or D.N.).

p_I is thus the same as the ionic yield for the reaction observed; k is a constant.

It follows from equation (3) that D_E, already defined, is given by—

$$D_E = \frac{A_O}{kp_I} + \frac{1}{k\tau Z_I p_R p_I} \quad . \quad . \quad . \quad . \quad (4)$$

which is of the same form as equations (1) and (2) obtained empirically for C.P. and D.N. Using the values of Z for C.P. (mol. wt. 35,000) (Dale *et al.*, 1949), and D.N. (mol. wt. 920) calculated by Lea (1496, p. 53), we find—

(1) the ionic yield, p_I for C.P. $= 0 \cdot 19$.

(2) the ionic yield, p_I for D.N. $= 0 \cdot 40$,

(3) the value of τ from the C.P. data $= 3 \cdot 4 \times 10^{-7} \left[\frac{1}{p_R}\right]_{C.P.}$ secs.,

and (4) the value of τ from the D.N. data $= 7 \cdot 5 \times 10^{-7} \left[\frac{1}{p_R}\right]_{D.N.}$ secs.

And therefore, since τ should be independent of the solute, we conclude that the probability (p_R) of a radical being destroyed when in collision with D.N. molecule is 2·2 times as great as the corresponding probability for C.P.

The protection effect.

Lea *et al.* (1944) also suggested a modification of equation (3) to cover the protection effect on the assumption that the indicator and protector molecules "share" the available radicals. We then have—

$$A/A_O = e^{-\frac{Z_I p_R p_I}{Z_I p_R A_O + \frac{1}{\tau} + Z_p p_p \cdot q} \cdot kD} \quad . \quad . \quad . \quad . \quad (5)$$

where Z_P is the collision frequency for the "protector," p_P the probability that a collision between a protector molecule and a radical destroys the latter, and q is the concentration of the "protector."

From equation (5) it follows that D_{P+E} is given by—

$$D_{P+E} = \frac{A_0}{kp_I} + \frac{1}{k\tau Z_I p_R p_I} + \frac{Z_P p_P q}{k Z_I p_R p_I} \quad . \quad . \quad . \quad (6)$$

whence in conjunction with equation (4) we find that—

$$Q = \frac{D_{P+E} - D_E}{D_E} \cdot \frac{A_0}{q} = \frac{A_0}{A_0 + m} \cdot l \quad . \quad . \quad . \quad (7)$$

$$\text{where } l = \frac{Z_P p_P}{Z_I p_R} \quad \text{and } m = \frac{1}{\tau Z_I p_R}.$$

Thus Q is independent of q, the concentration of the protector, and therefore it can be seen that whilst this simple theory is adequate for a number of protective substances, it does not fit those which exhibit the "changing quotient," which apparently involves some factor not included above.

However, equation (7) is valid for a large number of substances, and by means of it values of $\left(\frac{p_P}{p_R}\right)$, i.e. the ratio of the probabilities of the destruction of a radical by a collision with a protector and an indicator molecule respectively, can be obtained for these systems. $\left(\frac{p_P}{p_R}\right)$ is a measure of the protective power, per molecule, of the protector for the given indicator.

The "changing quotient."

This phenomenon implies a diminishing efficiency of the protective substance as its concentration is increased, and one explanation of this is to assume that a protector molecule may, after reaction with a radical, be in some state in which it can deactivate an indicator molecule with which it may collide. Thus although the protector molecule has removed a radical from the solution, it can "hand on" the effect of the radical to the indicator molecule, and thus fails to fulfil its function as a protector. Since we have no clue to the nature of this change we will call it "activation," and it might be thought of as a sort of meta-stable state. The only three ways open for a change back to stability would appear to be—

(1) By interaction with indicator molecules.

(2) By spontaneous change or by collision with solvent molecules with an average life τ_a.

(3) By interaction with non-activated protector molecules.

The existence of the "changing quotient" appears to exclude the third of these possibilities, and taking the other two into account we find that equation (5) is modified to become—

$$A/A_0 = e^{-\frac{(A_0 + T)p_I + lp_A p_N q}{(A_0 + T)(A_0 + m + lq)} \cdot kD} \quad . \quad . \quad . \quad (8)$$

and hence

$$kD_{P+E} = \frac{(A_O + T)(A_O + m + lq)}{(A_O + T)p_I + lp_A p_N q} \quad . \quad . \quad . \quad (9)$$

Where T is a constant containing τ_a and the collision frequency between "activated" protector molecules and indicator molecules, l, m and p_I have already been defined, whilst p_A is the probability that a collision between a radical and a protector molecule destroys the former and activates the latter. p_N is the probability of an indicator molecule being inactivated in a collision with an "activated" protector molecule.

From equations (9) and (4) we find—

$$Q = \frac{A_O}{A_O + m} \cdot l\left[\frac{(A_O + T)p_I - (A_O + m)p_A p_N}{(A_O + T)p_I + lp_A p_N q}\right] \quad . \quad (10)$$

Some of the constants appearing in equation (10) can be evaluated from results obtained with solutions of the indicator alone, whilst the others can be evaluated from any two experimental values of Q for known protector concentrations. Thus a calculated curve for the variation of Q with q can be produced and two examples are shown in Fig. 3, Curve A and B. The agreement is good, and so is the agreement between the experimental results obtained using a different indicator concentration (90 μg. C.P. per ml.) (Fig. 3, Curve C), and those deduced by means of the formula and the constants evaluated for 30 μg. C.P. per ml. A single experiment was performed with 150 μg. glucose per ml. protecting a solution of 270 μg. C.P. per ml. The predicted value of Q was $1{\cdot}5 \times 10^{-2}$, whilst the observed value was $1{\cdot}2 \times 10^{-2}$.

RESULTS.

The above formulae enable the values of $\left(\frac{p_P}{p_R}\right)$ to be calculated for any protector and indicator system, even when there is a "changing quotient."

TABLE III.—*Protective Power per Molecule of Various Substances Relative to C.P. and D.N.*

Protector.	Molecular weight.	$\left[\frac{p_P}{p_R}\right]$ with C.P.	$\left[\frac{p_P}{p_R}\right]$ with D.N.	$\frac{[p_R]_{D.N.}}{[p_R]_{C.P.}}$
Thiourea	76	4·7	2·1	2·2
Sodium formate	68	3·1	0·74	4·2
Dimethylthiourea	104	1·9	..	..
Glucose	180	0·79	0·21	3·8
Dimethylurea	88	0·4	..	..
Egg albumin	~40,000	0·24	0·21	1·1
Alloxan	142	$2{\cdot}1 \times 10^{-2}$		
Sodium mesoxalate	152	$2{\cdot}4 \times 10^{-2}$		
Sodium oxalate	134	2×10^{-3}		
Urea	60	$7{\cdot}5 \times 10^{-4}$		

Ratio of values of τ obtained from experiments with indicator only . 2·2

The protection of solutions of C.P. has been the subject of most of the investigations, whilst D.N. was used in a few cases.

In Table III are shown the values obtained from these experiments. The values of $\left(\frac{p_P}{p_R}\right)$ are indications of the protective power per molecule of each substance for the particular indicator, and the values given show the same tendency as was shown previously for protective power per unit weight by Dale (1947). Molecules with approximately the same atomic constitution have approximately equal protective powers, whilst marked differences occur when special groupings are present.

From the results it is also possible to calculate the relative values of p_R for D.N. and C.P., and quite good agreement is obtained from protectors of widely different constitution with the value obtained from the calculation of the value of τ.

SUMMARY.

Experiments are described on—

(1) The variation of the ionic yield of solutions of carboxypeptidase, and of alloxazin-adenine-dinucleotide, irradiated by X-rays.

(2) The specific protective action of unit mass of various solutes when irradiated—

(*a*) In presence of carboxypeptidase.
(*b*) In presence of alloxazin-adenine-dinucleotide.

(3) The variation of the protective power per unit mass of protector when the concentrations of protector and indicator are varied (phenomenon of the " changing quotient ").

In the discussion of the experiments, a quantitative interpretation of the protective power of various substances in terms of collision frequencies is given, which includes the phenomenon of the " changing quotient."

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URINARY CHOLESTEROL IN CANCER: URINARY CHOLESTEROL EXCRETION IN CANCER PATIENTS AND CONTROL SUBJECTS.

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THE application of the methods of urinary cholesterol estimation given by Burchell and Maclagan (1949) to cancer and control cases will now be described.

MATERIAL.

Urine specimens were obtained from 13 normal subjects and 116 patients admitted to Westminster Hospital or to Westminster Hospital (All Saints') Urological Centre. Twenty-five of these patients were suffering from diseases thought to be unlikely to affect cholesterol excretion as shown in the tables, and 73 were suffering from various forms of cancer. The remaining 18 were patients suffering from haematuria or pyuria of non-malignant origin. The diagnosis in the cancer group was established in most cases by laparotomy (3), biopsy (38) or autopsy (12), but in 20 cases the diagnosis was based on unequivocal X-ray or other clinical data.

Collection and treatment of specimens.

Preliminary results having shown that a considerable amount of cholesterol might be associated with the urinary sediment in certain cases, a routine procedure was adopted to standardize the treatment of the sediment.

Twelve-hour specimens of urine were collected into clean winchesters, the time of the collection being usually between the hours of 6 p.m. and 6 a.m.

The volume of specimen was measured, the urine adjusted to pH 5·0 with acetic acid and, if urates were present, warmed to 37° C. Cell counts on the *urinary deposit* (UD) were carried out by the method of Addis (1925) on a 10 ml. portion of the well-mixed specimen. For cholesterol estimation on the deposit a 500 ml. sample of the mixed specimen, or a smaller aliquot in the case of a concentrated urine, was centrifuged, the deposit washed once with normal saline and extracted directly with three portions of boiling acetone. The acetone extract was evaporated to dryness, and the residue extracted with petroleum ether for the determination of cholesterol as above.

The cholesterol content of the *supernatant urine* (SU) was determined on 200 ml. portions by the aluminium tungstate method described by Burchell and Maclagan (1949). For urines containing much protein smaller volumes were taken according to the amount of protein present.

RESULTS.

Control subjects.

The cholesterol content of the *supernatant urine* in 23 control subjects is shown in Fig. 1. These subjects included 9 normal persons and 14 patients convalescent from diseases thought to be unlikely to affect cholesterol excretion, such as hernia, appendicitis, fractures, etc.

It appears from this that the upper normal limit may be taken as 0·5 mg. per 12 hours, and that there is no significant difference between the range of excretion in men and women.

The cholesterol content and cell counts of the *urinary deposit* in 20 control subjects is shown in Fig. 2. These subjects included 9 normal persons and 11 convalescent patients as above.

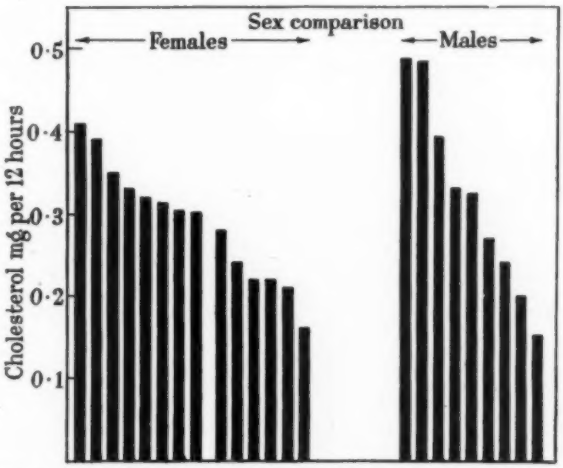

FIG. 1.—Cholesterol content. Supernatant urine. Twenty-three normal and control subjects.

It will be seen from this that the normal range both for cholesterol and for cell counts is higher for women than for men, presumably on account of the greater cellularity of the deposit from women. The upper normal limits for cholesterol were 0·05 mg. per 12 hours for men and 0·42 mg. for women. It will be noted that our figures for the normal cell counts are higher than those given by Addis (1926). The sex difference is mainly due to the larger number of epithelial cells and leucocytes in urinary deposits from women. It appears likely from these figures that the total urinary cholesterol would not exceed 0·9 mg. per 12 hours by our method, but as a result of our finding on the UD cholesterol we did not study total urinary cholesterol in normal subjects.

Albuminuria.

The association of albuminuria with increased cholesterol excretion is illustrated in Fig. 3.

It is evident from this that if albuminuria is present from whatever cause, the SU may be expected to show an increase in cholesterol content, and such a rise is unlikely to be specifically related to cancer. On the other hand, 4 patients excreted small amounts of protein without increased cholesterol. The correlation between urinary protein and cholesterol is definite, but not very close. Obviously the plasma cholesterol would influence this correlation, but no plasma cholesterol estimations were done on these patients.

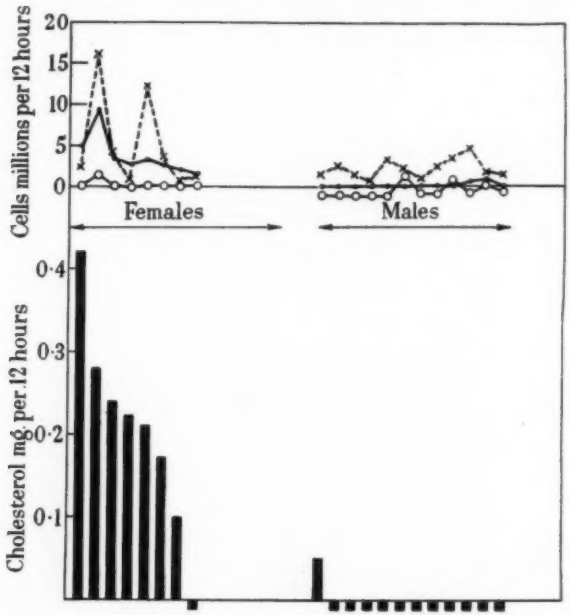

FIG. 2.—Cholesterol content of urinary deposits. Twenty normal and control cases.

× - - - - - × Leucocytes.
⊙ ——— ⊙ Red blood cells.
● ——— ● Epithelial cells.

Haematuria and pyuria.

Similarly, any patient with haematuria or pyuria may be expected to show an increase in the UD cholesterol as shown in Table I.

In this group of cases an attempt was made to correlate the cholesterol content of the UD with quantitative cell counts, and although no exact correlation could be established, it will be noted that the three highest cholesterol values were associated with the highest cell counts. Pyuria was the commonest accompaniment of raised UD cholesterol in this series, but increase in red cells and epithelial cells appear to be significant in cases No. 137 and 152.

It is evident from these results that a careful examination of the urinary deposit must accompany any cholesterol estimation if the result is to be

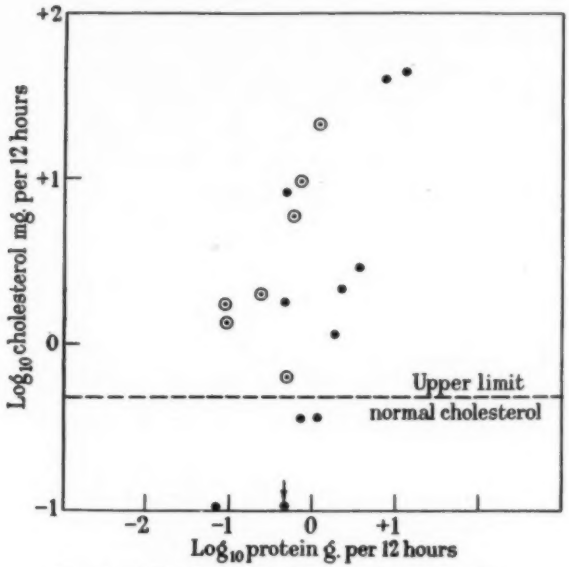

FIG. 3.—SU cholesterol in 17 cases of albuminuria.

⊙ Cancer cases.
● Non-cancer cases.

TABLE I.—*Cholesterol Content of Urinary Deposit in Cases of Haematuria and Pyuria.*

Case No.	Diagnosis.	Cholesterol (mg. per 12 hours).	Leucocytes (millions per 12 hours).	Red blood cells (millions per 12 hours).	Epithelial cells (millions per 12 hours).
151	Enlarged prostate	23·4	6,100	5,500	0
157	Carcinoma of bladder	6·35	314	12,700	0
165	Cystitis	4·35	1,420	380	0
166	Carcinoma of bladder	3·10	207	92	0·24
161	,, ,,	1·02	129	197	0
152	Ulcer of toe	0·98	100	0	5·90
156	T.B. cystitis	0·79	164	0	0
137	Chronic nephritis	0·72	1·7	4·4	0
144	Appendicectomy	0·71	196	0	2·84
119	Carcinoma of bladder	0·48	213	..	0·17

interpreted intelligently. Unless specimens are quite fresh, there is also the possible danger of decomposition and solution of blood cells to be considered.

Cancer.

The cholesterol content of the *supernatant urine* in 51 cases of various types of cancer is shown in Fig. 4, and full details of these patients are given in Table II. In these cases absence of albuminuria and pyuria was established by appropriate tests in each case.

It will be seen that only two cases were significantly above the normal level of 0·5 mg. per 12 hours, one case of carcinoma of oesophagus (No. 42, 0·68 mg.) and one of osteogenic sarcoma (No. 88, 0·73 mg.). There were three cases at border-line level (0·51–0·53 mg.), including one case of carcinoma of uterus, one of rectum and one of penis.

The cholesterol and cell content of the *urinary deposit* in 24 cases of cancer without albuminuria, haematuria or gross pyuria are shown in Fig. 5. Full details of these patients are given in Table III.

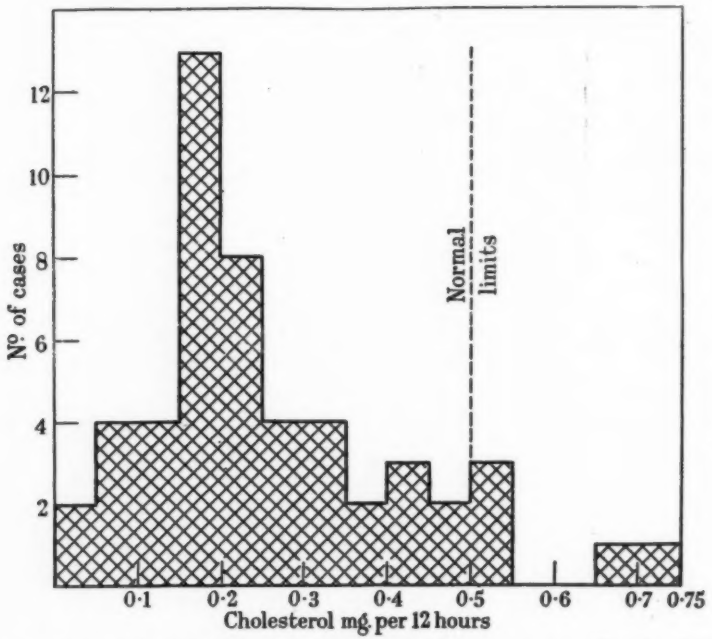

FIG. 4.—Cholesterol content of supernatant urine in 51 cancer cases. Albuminuria and pyuria excluded.

For the purpose of this table any leucocyte count in excess of 100 millions per 12 hours has been excluded. It will be seen from this table that no case showed cholesterol values significantly above the normal limits, although a marked sex difference is again obvious.

The UD cholesterol results in 4 cases of cancer with haematuria or pyuria (leucocyte counts above 100 millions per 12 hours) were shown in Table I, but it will be seen that the high values here were equally distributed between cancer and non-cancer cases, and were presumably due simply to an increase in cell content.

The cholesterol content of the *supernatant urine* in 11 cancer cases before and after treatment are shown in Table IV.

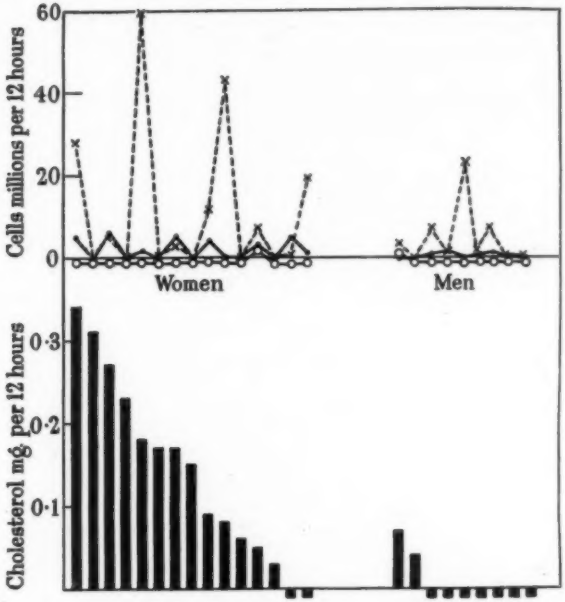

FIG. 5.—Cholesterol content of urinary deposits in 24 cancer cases. Albuminuria and pyuria excluded

× - - - - - × Leucocytes.
⊙ ——— ⊙ Red blood cells.
● ——— ● Epithelial cells.

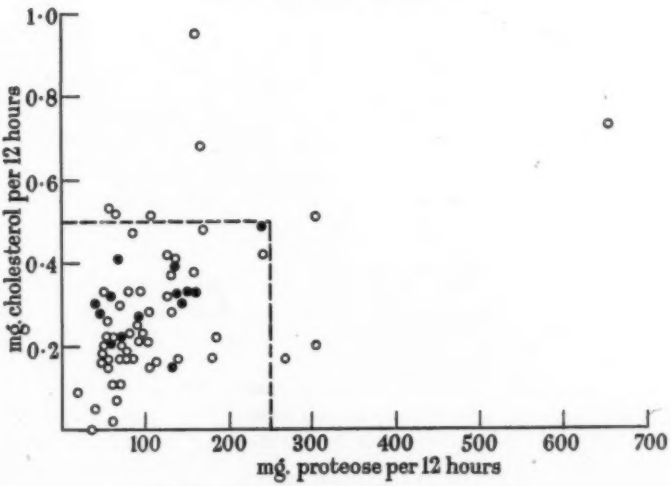

FIG. 6.—Urinary proteose and cholesterol.

● Control subjects.
○ Cancer patients.

TABLE II.—*Cholesterol Content of Supernatant Urine in Cancer Patients. Albuminuria and Pyuria Excluded.*

Case No.	Sex.	Diagnosis.	Mg. cholesterol per 12 hours.
88	M.	Osteogenic sarcoma	0·73
42	M.	Carcinoma of oesophagus	0·68
72	M.	,, penis	0·53
34	F.	,, uterus	0·52
73	M.	,, rectum	0·51
19	F.	,, breast, bone metastases	0·48
78	F.	,, cervix	0·47
77	M.	,, bladder	0·42
15	M.	,, bronchus	0·42
82	F.	,, breast, metastases in lung	0·41
89	F.	,, breast and secondaries	0·38
31	F.	,, colon	0·37
14	F.	Lymphadenoma	0·33
48	M.	Osteogenic sarcoma	0·33
71	F.	Carcinoma of breast	0·33
92	M.	Lymphadenoma	0·32
28	F.	Carcinoma of colon	0·30
40*b*	M.	,, bronchus	0·28
98	F.	Fungating carcinoma of breast	0·28
80	F.	Carcinoma of breast	0·26
55	M.	Fibrosarcoma of calf	0·25
17	F.	Secondary deposits after melanoma	0·23
105	M.	Carcinoma of bronchus	0·23
26	F.	,, rectum	0·22
41	F.	,, uterus, secondaries of ilium	0·22
65	F.	,, breast	0·22
87	F.	,, ,,	0·21
94	M.	Sarcoma of thigh	0·21
21	M.	Carcinomatous peritonii	0·20
27	F.	Carcinoma of oesophagus	0·20
83	F.	Paget's disease of nipple	0·20
40*a*	M.	Carcinoma of stomach	0·19
96	M.	Rodent of temporal region	0·19
22	M.	Carcinoma of bronchus	0·17
25	M.	,, oesophagus	0·17
29	F.	Primary carcinoma of rectum, multiple secondaries	0·17
62	F.	Carcinoma of breast and secondaries	0·17
106	M.	Rodent ulcer	0·17
108	F.	Epithelioma of ear	0·17
18	F.	Secondaries in spine from carcinoma of breast	0·16
104	F.	Carcinoma of breast and secondaries	0·16
30	M.	,, palate, tonsil and pharynx	0·15
95	M.	,, larynx	0·15
43	F.	,, breast and glands	0·11
75	M.	,, parotid gland	0·11
52	M.	,, oesophagus	0·09
10	M.	Multiple secondaries	0·08
13	M.	Carcinoma of stomach	0·07
20	M.	Secondary deposits after melanoma	0·05
107	M.	Epithelioma of vocal cord and arytenoid process	0
61	F.	Carcinoma of ovary	0

It will be seen that there was no constant change, 7 cases showing a rise and 4 showing a fall after treatment. All values are within normal limits.

Proteose and cholesterol content of supernatant urines.

The relationship between the SU cholesterol and SU proteose for 14 control and 53 cancer cases is shown in Fig. 6.

TABLE III.—*Cholesterol Content of Urinary Deposits in Cancer Cases. Albuminuria and Pyuria Excluded.*

Case No.	Sex.	Diagnosis.	Mg. cholesterol per 12 hrs.	Leucocytes (millions per 12 hrs.).	Red blood cells (millions per 12 hrs.).	Epithelial cells (millions per 12 hrs.).
136	F.	Carcinoma of ovary	0·34	28·0	0	4·50
90	F.	,, breast, secondary deposits	0·31	—	—	—
124	F.	,, stomach	0·27	5·06	0	3·68
83	F.	Paget's disease of nipple	0·23	—	—	—
118	F.	Carcinoma of breast. Secondaries	0·18	58·50	0	0·27
98	F.	Fungating carcinoma of breast	0·17	—	—	—
133	F.	Carcinoma of breast	0·17	3·16	0	4·44
97	F.	,, uterus	0·15	—	—	—
131	F.	,, breast, secondary of femur	0·09	11·7	0	2·0
117	F.	,, cervix	0·08	42·5	0	0
87	F.	,, breast	0·06	—	—	—
125	F.	,, rectum	0·05	6·2	1·46	0·88
82	F.	,, breast, metastases in lung	0·03	—	—	—
114	F.	,, ,,	0	0	0	4·55
122	F.	Anal carcinoma	0	19·5	0	0·34
134	M.	Malignant tumour of tibia	0·07	2·6	0·20	0
92	M.	Lymphadenoma	0·04	—	—	—
121	M.	Multiple metastases	0	6·15	0	0·13
130	M.	Carcinoma of tonsillar fossa and palate	0	0	0	0·17
116	M.	,, lung	0	22·2	0	0
142	M.	,, stomach	0	0·59	0	0·13
123	M.	Leukoplakia	0	6·85	0	0·38
99	M.	Sarcoma of thigh	0	—	—	—
88	M.	Osteogenic sarcoma	0	—	—	—

In cases where cell counts are not shown, the urinary deposit showed absence of abnormal constituents.

TABLE IV.—*Comparison of Cholesterol Contents of Supernatant Urine in 11 Cancer Patients Before and After Treatment.*

Case No.	Sex.	Diagnosis	Treatment.	Cholesterol (mg. per 12 hrs.). Before treatment.	After treatment.
71	F.	Carcinoma of breast	Irradiation	0·33	0·06
88	M.	Osteogenic sarcoma	,,	0·73	0·15
94	M.	Sarcoma of thigh	,,	0·21	0·34
95	M.	Carcinoma of larynx	,,	0·15	<0·10
97	F.	,, uterus	,,	0·51	0·37
96	M.	Rodent ulcer	,,	0·19	0·43
105	M.	Carcinoma of bronchus	,,	0·23	0·38
106	M.	Rodent ulcer	,,	0·17	0·19
108	F.	Epithelioma of right ear	,,	0·17	0·20
98	F.	Fungating carcinoma of breast	Radical mastectomy	0·28	0·37
104	F.	Carcinoma of breast	Testosterone	0·16	0·38

The proteose content of these urines was determined by treating the washed aluminium tungstate precipitate from 5 ml. of urine with phenol reagent as described by Burchell and Maclagan (1949), where reasons are given for assuming that this method does, in fact, estimate a proteose-like substance.

It will be seen from Fig. 6 that there is very little correlation between proteose and cholesterol, although the few cases outside normal limits with both estimations are cancer cases. Thus accepting the normal limits shown in Fig. 6, all 7 cases with high cholesterol values and all 4 cases with high proteose values were suffering from cancer. However, only 2 of these cases had both proteose and cholesterol above normal.

DISCUSSION.

One of the principal results of this study is to emphasize the essential difference between the nature of the cholesterol in the urinary deposit (UD) and in the supernatant urine (SU). The UD cholesterol is associated with the cells of the deposit and shows a marked sex difference, being much higher in women than in men. It appears to have no direct relation to cancer. The SU cholesterol on the other hand is associated with a urinary proteose fraction, or with heat-coagulable protein when present. Raised SU cholesterol excretion in the absence of albuminuria did occur in a small proportion of cases of cancer (5 out of 51 cases), although the increase was slight in 3 of these cases. This gives a total incidence of hypercholesteroluria in cancer of 9·8 per cent, which is somewhat lower than that reported by other workers. Thus Sobotka, Bloch and Rosenbloom (1940) found 20·6 per cent, and Bruger and Ehrlich (1943) 34·3 per cent of positive results. Our highest values for total urine cholesterol in control subjects are about 0·9 mg. per 12 hours, and correspond approximately to those given by Sobotka, Bloch and Rosenbloom for pooled specimens on non-cancer subjects (up to 0·5 mg. per litre). They are, however, appreciably lower than Bruger and Ehrlich's normal upper limit of 3·88 mg. per 24 hours.

It is evident that the accidental inclusion of cases with albuminuria or pyuria is a great danger in this work, as the former will cause a rise in the SU cholesterol, and the latter a rise in the UD cholesterol. We feel therefore that urinary cholesterol values are difficult to interpret without quantitative data on urine protein and deposit, and it is possible that the omission of this precaution by other workers may explain the apparent discrepancy between their results and ours. For example, Bruger and Ehrlich (1943) reported some rise of total urinary cholesterol in 11 out of 32 cases of cancer, but 5 of these had either albuminuria or excess of cells in the deposit or both, findings which, according to our experience, might have been sufficient to account for the hypercholesteroluria even in the absence of cancer. The exclusion of these cases would bring the incidence of positive results down to the figure of 22·2 per cent., which does not differ significantly from our own finding of 9·8 per cent (difference 12·4, standard error of difference 8·2).

We must conclude, therefore, that hypercholesteroluria, while frequently due to associated albuminuria or pyuria, does occur in a small proportion of cases of cancer in the absence of these factors. In such cases it is the supernatant urine cholesterol which is increased. The incidence is not high enough to make the estimation of any diagnostic value, but it is of theoretical interest, and presumably depends upon increased tissue breakdown as suggested by previous workers. It should be noted that the colorimetric method of estimation used is not entirely specific for cholesterol, and the possibility that other steroids might have given rise to the few positive results must be admitted. However, the earlier work of Bloch and Sobotka (1938) on pooled 100 litre specimens of urine suggests that the results probably were due to increase of cholesterol itself.

The question of urinary proteose is of some interest in view of the work of Winzler and Burk (1943) on blood proteose and cancer in rats. Our observations on the urinary proteose estimated by the aluminium tungstate method include 4 cases of cancer with high urinary proteose values. This is again a small

proportion of the 53 cases tested. Only two of these patients also had high urinary cholesterol excretion, so that there appears to be an occasional association between cancer and urinary proteose, which is independent of cholesteroluria. Presumably this phenomenon also depends upon increased tissue breakdown in the tumour tissue.

SUMMARY.

1. Values are given for the 12-hourly excretion of urinary cholesterol in 38 control subjects and in 73 patients suffering from various forms of cancer.

2. The cholesterol in the urine deposit (UD) normally varies from 0–0·05 mg. per 12 hours in men, and from 0–0·42 mg. in women. The supernatant urine cholesterol (SU) varies from 0–0·5 mg. per 12 hours with no sex difference.

3. Albuminuria if present increases the SU cholesterol, haematuria or pyuria increases the UD cholesterol.

4. In 51 cases of cancer without albuminuria the SU cholesterol was above normal in 5, 3 of these cases having only slightly raised values.

5. In 24 cases of cancer without haematuria or pyuria the UD cholesterol was within normal limits.

6. In 53 cases of cancer without albuminuria, the SU proteose was above normal in 4 cases.

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URINARY CHOLESTEROL IN CANCER: CHEMICAL STATE OF URINARY CHOLESTEROL AND METHODS OF ESTIMATION.

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DURING recent years two groups of workers have reported a notable increase in urinary cholesterol excretion in cancer. Bloch and Sobotka (1938) worked with pooled urine specimens from cancer wards and compared them with pooled specimens from cardiac and tuberculosis wards, and found approximately ten times as much cholesterol in the cancer urine as compared with the cardiac and tuberculosis urine. In this study the cholesterol was isolated in relatively large quantities (0·35 g. per 100 litres of urine), and its identity was proved by determination of melting-point, optical rotation and preparation of the acetate.

This work was later extended by Sobotka, Bloch and Rosenbloom (1940) to individual cases of cancer using a less specific colorimetric method, depending on the Liebermann-Burchard reaction. They reported an increase of urinary cholesterol in 19 out of 92 cancer patients. Later, Bruger and Ehrlich (1943), using a similar method of estimation, found a higher incidence of hypercholesteroluria, 11 of their 32 cases giving results above normal limits. However, the actual concentration of cholesterol reported by Bruger and Ehrlich in normal urine was approximately twice as great as that found by Sobotka, Bloch and Rosenbloom. It appeared, therefore, that the methods used in these two studies must have been significantly different, and we felt that further work on the technique of estimation was needed. While admitting the possible non-specificity of the Liebermann-Burchard reaction, we felt that this type of method was the only one likely to find immediate clinical application, and it does appear to have given results consistent with the original work of Bloch and Sobotka. The present work was undertaken with the main object of finding out whether refinements of this colorimetric method of estimation would be of any value in the diagnosis of cancer.

EXPERIMENTAL.

Extraction method.

The chloroform extraction method of Neustadt, Howard and Myerson (1946) was first tried, but the amount of colour obtained at the final stage from the normal urines was too small for accurate measurement. Adaptation of this method, using the whole of the extract from 200 ml. of urine (in place of 50 ml.), gave a brown rather than a green colour with the Liebermann-Burchard reagent, as shown in Fig. 1. An attempt was made to correct for this error by the introduction of a blank reading, which was obtained by adding to half the urinary extract in 5 ml. of chloroform 0·9 ml. of ethanol and 0·1 ml. of concentrated H_2SO_4 in place of the Liebermann-Burchard reagent. This sometimes gave

appreciable readings up to the equivalent of 0·15 mg. of cholesterol when compared in the photoelectric absorptiometer (King-Gallenkamp) with the Ilford spectrum red filter (maximum transmission 660mμ.). As an alternative to charcoal treatment a further stage was introduced, which consisted of evaporating the chloroform solution to dryness and extracting the residue with several portions of hot petroleum ether (B.P. 40–60° C.) before final colorimetry, but the blank values were still as high as 0·06 mg. of cholesterol in some cases—a value comparable with the amount in 100 ml. of normal urine. A further disadvantage of the extraction method was the occasional difficulty in separating the emulsion produced during the preliminary extraction with chloroform.

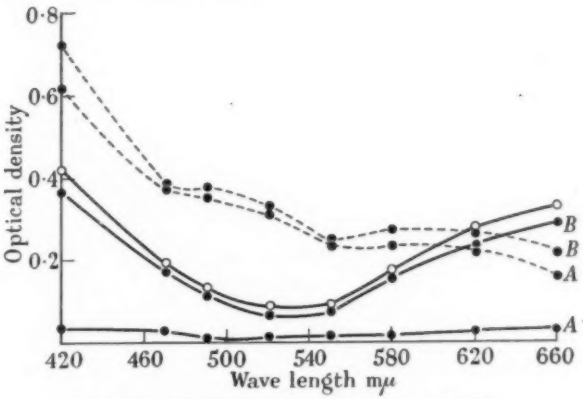

FIG. 1.— Absorption spectra of urinary extracts.
A. Urinary extract.
B. Urinary extract with added cholesterol.
Extraction method.
Aluminium tungstate method.
Standard solution.

We therefore felt that this procedure, although apparently giving good recoveries of added cholestesol of the order of 0·5 mg., is not suitable for the estimation of the small amounts found in normal urine. Attempts were therefore made to find an alternative method which would yield a final solution uncontaminated by urinary pigments.

Adsorption methods.

Numerous experiments were made on urine with added free cholesterol and/or plasma to determine whether the cholesterol could be completely removed from the urine by adsorption methods. The general procedure was to filter the urine slowly through a ¾-inch wide column of adsorbent from 1 to 3 inches in length, and after washing the column with water, to elute with 40 ml. of cold acetone. The acetone was then evaporated to dryness, the residue extracted with several portions of hot petroleum ether, and the petroleum ether solution evaporated to dryness. The final residue was then dissolved in 5 ml. of chloroform and

treated with the Liebermann-Burchard reagent as described below under aluminium tungstate method. The final chloroform solution was invariably colourless, and gave negligible blank values when treated with 0·9 ml. of ethanol and 0·1 ml. of concentrated H_2SO_4 as described above. Table I gives some typical results illustrating the efficiency of the various adsorbents studied.

TABLE I.—*Recovery of Added Cholesterol from 100 ml. Urine by Means of Various Adsorbents.*

Absorbet.	Column.	Cholesterol added.	Per cent added cholesterol eluted from column.	Per cent added cholesterol in filtrate by extraction.
Alumina	1 × ¾ in.	1·06 mg. in 0·2 ml. plasma	62	27
,,	1 × ¾ in.	Ditto	79	7
,, charcoal	1 × 1 in.	,,	77	25
,, casein	1 × ¾ in.	,,	..	64
Charcoal	1 × ½ in.	1·0 mg. in acetone solution	43	*nil.*

It appears evident from these experiments that alumina was the most promising adsorbent, retaining up to 93 per cent of the added cholesterol, most of which was successfully eluted with acetone (79 per cent). Charcoal was a more efficient adsorbent (100 per cent), but the adsorbed cholesterol could not be eluted with acetone, and only incompletely eluted with chloroform. The recovery with alumina was, however, far from quantitative, and it soon became evident that this was connected with the amount of protein present in the urine, the proportion of protein removed by the column being very similar to that of cholesterol removed. This relationship is illustrated in Table II, which gives the result of an experiment in which a larger amount of plasma was added (2 ml. to 100 ml. of urine).

TABLE II.—*Effect of Adsorption by Alumina (3 × ¾ in. Column) on Urinary Cholesterol and Protein Content.*

Material : 100 ml. normal urine, 2·0 ml. plasma.

	Original urine.	Filtrate.	Per cent adsorbed.
Protein	115 mg.	84 mg.	27
Cholesterol	3·8 mg.	2·9 mg.	24
Ratio : Protein/cholesterol	30	29	..

In this experiment the cholesterol was estimated by chloroform extraction as above and the protein by a turbidometric method with salicylsulphonic acid precipitation. It will be seen that a similar proportion of urinary protein (27 per cent) and of urinary cholesterol (24 per cent) was removed by the column.

It appeared likely from these experiments that adsorption by alumina would be more efficient in the absence of protein, and Table III shows the recovery

obtained by adding cholesterol in acetone solution to urine previously deproteinized by the tungstic acid procedure of Bruger and Ehrlich (1942).

TABLE III.—*Recovery of Added Cholesterol from Proteose-free Urine by Means of Alumina* 1 *mg. Cholesterol Added to* 100 *ml. Samples. Alumina Columns* $3 \times \frac{3}{4}$ in.

pH.	Per cent added cholesterol recovered in eluate.	
3	74	Av. 85
3	74	
3	90	
1	87	
5	90	
13	92	

It should be mentioned that in this method a large excess of sulphuric acid is used, the final pH being about 3. Adjustment of the reaction after precipitation to pH 1, 5 or 13 as indicated in Table III did not improve the recovery, which averaged 85 per cent. Recovery from untreated urine on the other hand, as shown in Table IV, averaged only 72 per cent, the difference being presumably due to the presence of normal urinary proteose which is precipitated by the tungstic acid under these conditions.

TABLE IV.—*Recovery of Added Cholesterol from Normal Urine by Means of Alumina.*

Cholesterol added to 100 ml. samples of urine at pH5.

Mg. cholesterol added.	Per cent added cholesterol recovered in eluate.	
0·5	72	Av. 72
0·5	68	
1·0	76	
1·0	78	
1·0	68	

This adsorption method had the advantage of yielding colourless extracts free from interfering pigments, but was limited by the relationship with urinary protein and proteose noted above. It was, however, very useful as a check on the filtrates obtained by the various precipitation methods studied later.

Precipitation methods.

In view of the association between protein and cholesterol shown in these experiments, it appeared likely that methods involving protein and/or proteose precipitation would be more promising.

1. *Tungstic acid.*—The tungstic acid precipitation method of Bruger and Ehrlich (1942) was next tried without the addition of protein, but in our hands suffered from the following disadvantages: The precipitation was slow, and

accompanied by gradual deposition of uric acid, making the end-point difficult to determine. The large tarry residue after alcohol-ether extraction was difficult to extract with petroleum ether and recovery of added cholesterol was poor, ranging from 30 to 70 per cent in various experiments. In this method a gross excess of sulphuric acid is used which is difficult to wash out of the precipitate and results in charring. The precipitate obtained apparently consists of proteose and uric acid as well as protein when present. The use of smaller amounts of sulphuric acid frequently fails to produce any precipitate with normal urine.

2. *Tannic acid.*—Tannic acid in a 1 per cent W/V final concentration was used but recovery was again poor (32 per cent), apparently on account of direct destruction of cholesterol or interference with its extraction by alcohol-ether mixtures.

3. *Neutral precipitants.*—Zinc hydroxide, copper tungstate and aluminium tungstate were used, with the results shown in Table V. In these experiments the precipitates, after being washed with water, were extracted with boiling acetone, the extract evaporated to dryness, extracted with petroleum ether, evaporated to dryness and dissolved in 5 ml. of chloroform for final colorimetry. The extracts were in all cases colourless and final Liebermann-Burchard colour a pure green as shown by the absorption spectra in Fig. 1. The filtrates were put through an alumina column and worked up as described above. It will be seen from Table V that recovery was quantitative with aluminium tungstate, which moreover had the advantage of giving a less bulky precipitate than zinc hydroxide or copper tungstate.

TABLE V.—*Recovery of Added Cholesterol from Normal Urine by Means of Neutral Precipitants.*

Cholesterol added to 100 ml. samples.
Cholesterol in filtrates determined by adsorption methods.

Precipitant.	Mg. cholesterol added.	Per cent added cholesterol recovered from precipitate.	Cholesterol found in filtrate.
Zinc hydroxide	1·0	87	*nil*
Copper tungstate	1·0	83	,,
Aluminium tungstate	0·5	100	,,

Further experiments showed that the amount of washing of the aluminium tungstate precipitate had an important influence on recovery, and the best results were obtained by the following method:

Aluminium tungstate method.

To 200 ml. of urine were added 10 ml. of 10 per cent sodium tungstate followed by 10 ml. of 0·73M sodium aluminium sulphate, and the mixture left to stand for 5 to 10 minutes. The precipitate was collected by centrifugation and washed twice with successive portions of 60 ml. of hot water. The washed precipitate was extracted three times with boiling acetone, and the acetone removed from the combined extracts by distillation over a boiling water bath. The small aqueous residue was then evaporated to dryness in an oven at 105° C., the process being assisted by drawing a stream of air through the flask. The residue was extracted several times with hot petroleum ether (B.P. 40–60° C.), the extract

evaporated to dryness and the residue dissolved in 5 ml. of chloroform for final colorimetry. A standard solution containing 0·5 mg. of cholesterol in 5 ml. of chloroform was prepared for comparison at each estimation. 1 ml. of the Liebermann-Burchard reagent was added to each of the test and standard solutions, the treated solutions kept in the dark at 25° C. for 20 minutes, and the green colour compared in the photoelectric absorptiometer (King-Gallenkamp) with the Ilford spectrum red filter (maximum transmission 660mμ.) A preliminary calibration curve showed that Beer's law was well obeyed up to optical densities of 0·5. The cholesterol content of the urine was therefore calculated as follows :

$$\text{Urinary cholesterol in mg. per 100 ml.} = \frac{0{\cdot}25 \times \text{reading of unknown}}{\text{Reading of standard.}}$$

Recovery experiments.

Recoveries of added cholesterol from urine free from heat-coagulable protein by this method are shown in Table VI. It will be seen that recovery averaged 97 per cent with 100 ml. portions of urine and 87 per cent with 200 ml. portions. In spite of the slightly poorer recovery the latter volume was adopted in further work on account of the small amounts present in many of the specimens studied.

TABLE VI.—*Recovery of Cholesterol from Urine by Aluminium Tungstate Precipitation Method.*

0·5 mg. cholesterol added to each sample.

Volume of urine.	Mg. cholesterol in sample.	Mg. cholesterol recovered.	Per cent total cholesterol recovered.
100 ml.	0·04	0·55	102
,,	0·11	0·60	99
,,	Negligible	0·50	100
,,	,,	0·49	98
,,	0·10	0·53	88
200 ml.	0·06	0·41	73
,,	0·04	0·43	80
,,	0·06	0·58	103
,,	0·16	0·59	90
,,	0·10	0·53	89

Distribution of Cholesterol Between Urinary Deposit (UD) and Supernatant Urine (SU).

Table VII shows the distribution of cholesterol between UD and SU in 11 cases of cancer without albuminuria or gross haematuria. In these cases the urine was adjusted to pH5 and warmed to dissolve urates if necessary before centrifugation. The deposit was washed once with normal saline and extracted three times with boiling acetone. The acetone extract was then evaporated to dryness and the residue extracted with petroleum ether as above.

It is evident from this that there is a wide variation in different cases, and that the whole of the urinary cholesterol may be found either in the deposit or in the supernatant urine, even in the absence of any pathological deposit. Haematuria

TABLE VII.—*Distribution of Cholesterol Between Urinary Deposit and Supernatant Urine. (Haematuria and Pyuria Excluded.)*

Case number.	Sex.	Diagnosis.	Deposit.	Cholesterol. UD. mg. per 12 hrs.	SU. mg. per 12 hrs.	Total mg. per 12 hrs.	UD per cent of total.
109	F.	Carcinoma of tongue	Many epithelial cells	0·50	0	0·50	100
90	F.	Carcinoma of breast, secondary deposits	Ditto	031·	0·23	0·54	58
83	F.	Paget's disease of nipple	Few epithelial cells	0·23	0·20	0·43	54
98	F.	Fungating carcinoma of breast	Epithelial cells and few leucocytes	0·17	0·28	0·45	38
97	F.	Carcinoma of uterus	Leucocytes and epithelial cells	0·15	0·51	0·66	23
87	F.	Carcinoma of breast	Few epithelial cells	0·06	0·21	0·27	22
92	M.	Lymphadenoma	Occasional leucocytes	0·04	0·32	0·36	11
82	F.	Carcinoma of breast, metastases in lung	Ditto	0·03	0·41	0·44	7
114	F.	Carcinoma of breast	Few leucocytes	0	0·38	0·38	0
99	M.	Sarcoma of thigh	,,	0	0·34	0·34	0
88	M.	Osteogenic sarcoma	Occasional leucocyte	0	0·73	0·73	0

or pyuria, if present, causes a large increase in the UD cholesterol (Burchell, Earle and Maclagan, 1949).

CHEMICAL STATE OF CHOLESTEROL IN SUPERNATANT URINE.

1. *Proteose.*

The results given above under adsorption methods indicate a definite association between urinary cholesterol and protein when present, and suggest a possible association between cholesterol and proteose in normal urine. Further evidence of the latter association was sought by estimating the nitrogen content of the aluminium tungstate precipitate obtained (*a*) after washing the precipitate with water as above, and (*b*) after dialysis of the original urine before precipitation. It was assumed that any nitrogen remaining in the precipitate after these procedures was attributable to urinary proteose.

(a) *Washing of aluminium tungstate precipitate.*—The relationship between cholesterol and proteose was investigated by serial washing of the aluminium tungstate precipitate. In this experiment four identical samples of 100 ml. of normal urine with added cholesterol (0·5 mg.) were precipitated with aluminium tungstate, the precipitates were washed with varying numbers of 30 ml. portions of hot distilled water and the cholesterol determined as above. Similarly, four identical samples of 20 ml. of the urine were precipitated, washed with 6 ml. portions of hot distilled water, and the nitrogen content of the precipitate determined by the Kjeldahl method. The protein content was then calculated from the nitrogen figure, using a factor of 6·25. The results shown in Table VIII indicate that with increased washing the apparent cholesterol content of the

precipitate rises while the nitrogen content falls, there being little further change after four washings.

TABLE VIII.—*Cholesterol and Proteose Content of Aluminium Tungstate Precipitate from Urine after Serial Washing of the Precipitate.*

0.5 mg. cholesterol added to 100 ml. samples of normal urine.

Number of washings.	Mg. of proteose per 100 ml. of urine.	Mg. cholesterol recovered.
1	23	0·30
2	10	0·39
4	6·6	0·46
8	6	0·47

We have assumed that the nitrogenous compounds remaining after this washing procedure were of the nature of proteose. Some support for this assumption was obtained by treating the washed precipitate with biuret reagent (Hiller, 1927) and also with the phenol reagent of Folin and Ciocalteu (1927) as follows: The precipitate from 5 ml. of urine was suspended in 0·5 ml. dilute phenol reagent and 0·4 ml. 0·1 N HCl, followed by the addition of 2·5 ml. N NaOH and diluted to 10 ml. with water. A blank precipitate obtained by adding aluminium tungstate reagents to 5 ml. of water was suspended in 0·5 ml. of the phenol reagent and 0·4 ml. of standard tyrosine solution (200 mg./l in 0·1 N HCl) and treated as above. The blue colours were compared in the photoelectric absorptiometer after 5 minutes with the spectrum red filter.

The amount of proteose indicated by these tests was of the same order as that calculated from the nitrogen figure, as shown in Table IX.

TABLE IX.—*Comparison of Methods for Determination of Proteose Content of the Aluminium Tungstate Precipitate from Urine after Serial Washing of the Precipitate.*

Number of washings.	Mg. proteose/100 ml. of urine.		
	Biuret method.	Phenol method.	Kjeldahl method.
1	10	(23)	(17·4)
2	12	10	12·1
4	6	6·6	9·0
8	8	6·0	11·5

The improved recovery of cholesterol after washing noted above is very evident in this experiment. We assume that this is due to mechanical interference with acetone extraction by substances present on the precipitate which are removed by washing.

(b) *Dialysis.*—100 ml. of normal urine with added cholesterol (0·5 mg.) were dialysed in a collodion sac against running tap-water for 16 hours. Duplicate samples were similarly dialysed for 40 and 64 hours. The contents of each sac were then made up to 140 ml. with distilled water and the cholesterol content determined on 100 ml. as described above. A further 20 ml. of the dialysed urine were precipitated with aluminium tungstate, and the nitrogen content of the precipitate determined by the Kjeldahl method as above, without further washing. From the result of this experiment, as shown in Table X, it can be seen that the

non-dialysable proteose and cholesterol both reached a constant level after dialysis for 40 hours.

TABLE X.—*Cholesterol and Proteose Content of Urine after Dialysis.*

0·5 mg. cholesterol adeed to 100 ml. samples of normal urine.

Time of dialysis (hours).	Mg. proteose per 100 ml. of original urine.	Mg. cholesterol recovered.
16	7·4	0·40
40	6·2	0·44
64	6·2	0·44

Very little cholesterol dialysed out of the urine. These results are consistent with an association between the urinary cholesterol and non-dialysable proteose similar to that shown above between the urinary cholesterol and proteose determined after washing of the aluminium tungstate precipitate.

The final values for proteose and cholesterol obtained in the dialysis experiment (Table X) and in the washing experiment (Table VIII) correspond closely.

2. *Heat coagulable protein.*

The increase of urinary cholesterol accompanying proteinuria is well known (Gardner and Gainsborough, 1925; Bruger, 1935), but we were also interested in determining the distribution of native cholesterol and of added cholesterol between the heat-coagulable protein and proteose fractions. The results of a typical experiment are shown in Table XI. These were obtained by heat coagu-

TABLE XI.—*Distribution of Native Cholesterol and Added Cholesterol Between Heat-coagulable Protein and Proteose Fractions.*

Sample.	Mg. protein/ 100 ml.	Mg. cholesterol/100 ml.	
		Heat coagulable protein.	Centrifugate.
100 ml. urine	110	0·27	0·18
100 ml. urine 0·5 mg. cholesterol	110	0·77	0·17

lation after the addition of 0·5 ml. glacial acetic acid to 100 ml. of urine containing 110 mg. protein per 100 ml. The precipitated protein was removed by centrifugation, washed once with 0·5 per cent acetic acid and extracted three times with boiling acetone, the acetone extract evaporated to dryness and the residue extracted with petroleum ether for cholesterol determination as above. The proteose was precipitated from the combined centrifugate and washing by means of aluminium tungstate and the cholesterol content of the precipitate determined as above.

It is evident from the results that the heat-coagulable protein does not carry down the whole of the native cholesterol, but does remove all added cholesterol.

DISCUSSION.

In view of the insoluble nature of cholesterol in water it is evident that it must be held in solution in supernatant urine by some other substance, and the well-known association between protein and cholesterol in blood serum suggested

a similar association in urine. The experiments reported give some support to the hypothesis that the urinary cholesterol is normally associated with a urinary proteose fraction, and also with heat-coagulable protein if present. Removal of heat coagulable protein does not, however, carry down the whole of the native cholesterol.

Urine is not a homogeneous substance, and the varying distribution of cholesterol between the deposit and supernatant urine which we have demonstrated is obviously relevant to clinical studies. This factor has been recognized previously, e.g. Bruger in 1935 reported up to 40 per cent of the cholesterol in the urinary deposit in cases of nephritis. However, most authors have not recognized its importance, and have included the deposit even though pathological in certain cases (Sobotka, Bloch and Rosenbloom, 1940 ; Bruger and Ehrlich, 1943). Since either all or none of the urinary cholesterol may be found even in apparently normal deposits, it is evidently desirable to study the deposit and supernatant urine separately.

The aluminium tungstate method which we have developed appears to be suitable for clinical work on supernatant urine and avoids most of the disadvantages of previous methods. Results in cancer and control cases are given by Burchell, Earle and Maclagan (1949).

SUMMARY.

1. Reasons are given for doubting the specificity of certain previous extraction and precipitation methods of estimating urinary cholesterol.

2. Adsorption on alumina was successfully employed in the estimation of the cholesterol in de-proteinized urine, but did not recover the whole of the cholesterol from normal untreated urine.

3. A new method of estimation depending on aluminium tungstate precipitation has been developed which is suitable for normal supernatant urine.

4. Experiments involving adsorption, aluminium tungstate precipitation and dialysis indicate an association between the supernatant urine cholesterol and a proteose fraction.

5. Much or all of the urinary cholesterol may at times be found in the urinary deposit, or associated with heat-coagulable protein when present.

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A QUALITATIVE STUDY OF URINARY 17-KETOSTEROIDS IN NORMAL MALES AND IN MEN WITH PROSTATIC DISEASE.

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IN an earlier publication (Robinson, 1948) the results obtained in a study of 17-ketosteroid excretion in men of different age-groups were reported. Special attention was paid to quantitative differences in total ketosteroid excretion during the later decades of life. The primary object of the work was to form a basis for comparison with similar quantitative data from cases of prostatic cancer.

In parallel with this quantitative study a qualitative examination of these urines was also undertaken. This involved separation and estimation of the individual components of the total ketosteroid complex present in urinary extracts. Such a separation can be achieved by chromatographic methods, and many investigators have described procedures (e.g. Callow, 1939; Wolfe, Fieser and Friedgood, 1941; Dobriner, Rhoads, Lieberman, Hill and Fieser, 1944; Dobriner, Lieberman and Rhoads, 1948). In most of the work described by these authors the components were recognized by isolation and identification by the usual chemical criteria such as melting-point and optical rotation. Comparatively large quantities of material are required for identification by isolation, and the method is unsuitable for the amounts obtained from small specimens of urine.

Dingemanse, Huis in't Veld and de Laat (1946) described a method for chromatographic-colorimetric determination of urinary 17-ketosteroids which is applicable to the amounts encountered in one day's output of urine. The method involves adsorption of the 17-ketosteroids on a column of alumina from benzene solution followed by fractional elution with benzene of increasing ethanol content. The amount of 17-ketosteroid present in each eluate is determined by the Zimmermann reaction. Preliminary experiments with the method described by these authors showed that separation and estimation of the steroid components could be achieved, but that reproducible results depended on careful standardization of the alumina and of the conditions of elution. With these precautions this method was adopted in the present work.

METHODS AND MATERIALS.

(1) *Preparation of urine extracts.*

Hydrolysis and extraction of the urines were carried out by the same method as that given in the earlier paper (Robinson, 1948). For the chromatograms 5 mg. of total 17-ketosteroid was found to be the most suitable amount, and sufficient urine from a 24 or 48-hour collection was hydrolysed and extracted to furnish a quantity in excess of 5 mg. The required volume of urine was estimated approximately by a preliminary assay of total 17-ketosteroid on a small aliquot

of the urine. The bulk extract was also dissolved in alcohol and assayed for total ketosteroid. An amount of alcoholic solution containing about 5 mg. was evaporated to dryness, and two successive portions of pure benzene (5 ml.) added and distilled off to remove the last traces of alcohol prior to solution in benzene for adsorption on alumina.

(2) *Standardization of alumina and adsorption columns.*

Since Merck's alumina was not available, many samples of alumina were examined in order to obtain a product with the desired activity. Spence " Type H " alumina was finally selected for this purpose. When samples of this alumina are exposed to air (by spreading the material in thin layers and exposing it to the moist air of the laboratory) they gradually lose their adsorptive activity. This loss of activity is accompanied by a decrease in pH of aqueous suspensions of the alumina. Suspensions prepared by mixing alumina with distilled water (1 part Al_2O_3 with 2 parts distilled water, by weight) show an initial pH of 10·5 before deactivation and a gradual decrease to pH 9·3 after 2 to 3 days' deactivation. The adsorptive power of the alumina was checked at intervals during the deactivating process by employing Brockman's method of standardization, using mixtures of dyes (Brockmann and Schodder, 1941). A suitable dye mixture consists of 20 mg. of Sudan red together with 20 mg. of Sudan yellow dissolved in 10 ml. of benzene and diluted to a total volume of 50 ml. with petroleum ether (b.p. 40–60°). The dyes were obtained from Messrs. Gurr, and further purified by solution in benzene and filtration through alumina followed by crystallization from benzene (Sudan red) or alcohol (Sudan yellow). For standardization, 1 ml. of this dye solution is adsorbed on a column (50 × 14 mm.) prepared from a suspension of alumina (7 g.) in benzene-petroleum ether (1:4) and eluted with 30 ml. of the same solvent mixture. The alumina is considered to have suitable adsorptive power when the yellow band reaches the bottom of the column, while the red band moves down about 10 mm.

The alumina columns for adsorption of the urine extracts are contained in glass tubes of 14 mm. internal diameter fitted with glass taps at the lower end. Above the tap a plug of cotton-wool is inserted and the alumina filling is added above the plug. Each adsorption column contains exactly 13 g. of alumina, which forms a column approximately 10 cm. high. The dead space above the alumina is sufficient to hold 60–70 ml. of solvent. The column of alumina is formed by pouring a mixture of alumina and benzene into the tube and allowing packing of the alumina to take place by gravity alone. Excess benzene is drawn off through the tap until the benzene level reaches the top of the alumina; the tap is then closed and the column stoppered until required.

(3) *Adsorption and elution of urine extracts.*

The urine extract (containing approximately 5 mg. of total 17-ketosteroids) is dissolved in 50 ml. of dry thiophene-free benzene and the solution poured on to the prepared alumina column. The benzene eluate comprises Fraction O. Elution is then continued with (*a*) 350 ml. of benzene, (*b*) 1200 ml. of benzene containing 0·1 per cent. (v/v) ethanol, (*c*) 500 ml. of benzene containing 0·5 per cent (v/v) ethanol, (*d*) 100 ml. of ethanol. These solvents are passed through the column successively, the tube above the alumina being kept full (except

when the composition of the eluting fluid is changed), and the eluates are collected in 50 ml. fractions. These fractions are numbered successively 1–43.

(4) *Assay of eluted fractions.*

The solvent is removed from each fractional eluate and the residue dissolved in 2 ml. of absolute ethanol. An aliquot (0·2 ml.) of this solution is then assayed by the Callow-Zimmermann method for 17-ketosteroids. The observed colorimetric values are corrected when necessary for interfering chromogens (Robinson, 1948).

(5) *Recording of chromatograms and identification of constituents.*

The amount of 17-ketosteroid in each eluate is plotted as the ordinate against an abscissa corresponding to the serial number of the eluate. The curve constructed from these points consists of a series of peaks. With normal urine as many as nine peaks may occur, but in no case was the number less than seven.

Dingemanse, Huis in't Veld and de Laat (1946) adopted a system of numbering in which successive peaks on the curve were indicated by Roman numerals I–VIII, the lowest numeral corresponding to the peak with the lowest eluate serial numbers. This system was used in the present work to facilitate comparison with the results of these authors. In some urines, however, small quantities of a component occurred which appeared to be different from any of the 8 main fractions, and this component was allocated to a sub-group II*a*. The Dutch workers considered the chief 17-ketosteroid components of their 8 fractions to be (Huis in't Veld, 1948):

Fraction I	$\Delta^{3:5}$-androstadien-17-one.
	3-chloro-Δ^{5}-androsten-17-one.
	Δ^{2}-androsten-17-one.
,, II	" 17-ketosteroid II "
,, III	dehydro*iso*androsterone and *iso*androsterone (i.e. 3-(β)-hydroxyketosteroids)
,, IV	androsterone.
,, V	aetiocholan-3-(α)-ol-17-one.
,, VI	unidentified.
,, VII	,,
,, VIII	,, artefacts.

The substances in Fraction I are considered to be artefacts arising from the action of boiling hydrochloric acid during the hydrolysis. The substance originally referred to as " 17-ketosteroid II " (Dingemanse, Huis in't Veld and de Laat, 1946; Dingemanse, Huis in't Veld and Hartogh-Katz, 1948*a*) was later shown to be *i*-androsten-6-ol-17-one (Dingemanse, Huis in't Veld and Hartogh-Katz, 1948*b*; Barton and Klyne, 1948). The Dutch workers isolated this substance from the urine of a two-year-old girl with an adrenal tumour, but found only small quantities in the urine of normal persons. In the presence of hydrochloric acid this substance gives rise to chlorodehydro*iso*androsterone and dehydro*iso*androsterone, and such a conversion may occur during the preliminary hydrolysis of the urine.

In the present investigation confirmation of these identities for peaks III, IV and V was obtained (*a*) by the isolation of the constituents of peaks IV and V and the preparation of crystalline acetates and benzoates therefrom, (*b*) by the

application of a colour reaction (Patterson, 1947) to the constituents of peak III, (*c*) by identification of the peaks formed by authentic specimens of dehydro-*iso*androsterone, androsterone and aetiocholan-3-(α)-ol-17-one.

In order to study the reproducibility of the method and the type of curves to be expected, known amounts of dehydro*iso*androsterone, androsterone and aetiocholan-3-(α)-ol-17-one were chromatographed alone and in mixtures containing varying proportions of these three steroids. The curves obtained in these experiments revealed immediately a problem that has to be faced in determining the amount of ketosteroid represented by any one peak. This is the complication introduced by the fact that the minimum values in the depressions between peaks are not always zero. The question therefore arises as to how the amount of ketosteroid corresponding to such minimum values should be partitioned between two peaks on either side of the minimum. Three treatments were considered: (i) half of the minimum value might be allotted to each of the adjacent peaks; (ii) the whole of the minimum value might be added to the preceding peak; (iii) the descending curve of the preceding peak might be projected down to the abscissa axis, and the additional values thus formed added to the preceding peak while corresponding deductions are made from the succeeding peak. These three methods were tested by their application to the model chromatograms obtained with the known mixtures of the three steroids mentioned above. In general it was found that the projection method (iii) gave a closer approximation to the actual amounts of the individual steroids than did either method (i) or method (ii). This method was therefore adopted as standard for computing the amounts of 17-ketosteroids represented by different peaks.

A striking example of the reproducibility of the method has recently been encountered. Two specimens of urine from the same subject were examined at an interval of one year. The two curves were almost identical.

RESULTS.

Urines of 19 normal males varying in age from 3 to 73 years were examined. Representative curves obtained from some of these urines are shown in Fig. 1. The curve of Case 0 was obtained on an extract from a 10-day collection of urine from a boy aged three. In Cases 1 and 2 it was necessary to collect urine for 2 to 3 days in order to obtain sufficient ketosteroid for analysis. The remaining curves were obtained on aliquots of single 24-hour specimens. The percentage distribution of the total ketosteroids among the different peaks is given in Table I for all the normal males investigated with the exception of Case 0, in which the peaks normally present in adult curves could not be differentiated.

Urines from 2 cases of benign hyperplasia of the prostate and from 6 cases of carcinoma of this gland were also examined, and the corresponding curves and percentage compositions of these extracts are shown in Fig. 2 and Table II.

DISCUSSION.

Chromatographic analysis of the 17-ketosteroids present in normal or pathological urines usually reveals a complicated pattern. Part of the complication undoubtedly arises owing to the formation of artefacts during hydrolysis. In some cases investigated as many as 9 peaks appeared on the curves, indicating at least this number of different constituents. Even more components may,

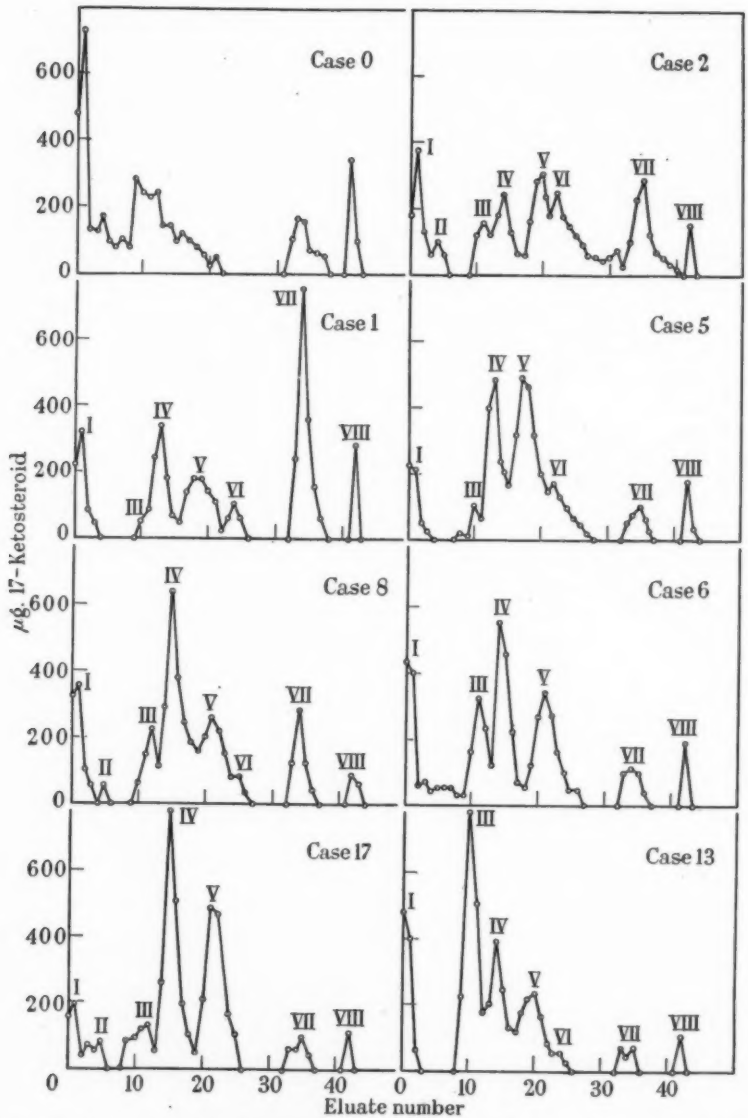

FIG. 1.—17-Ketosteroid content of eluates from chromatograms of urines from normal males.

Case 0 (not included in Table I). Three-year-old boy. Incomplete differentiation into individual peaks.

Cases 1, 8, 17 represent the predominant type in which androsterone exceeds aetiocholanolone.

Cases 2 and 5 are unusual types in which aetiocholanolone exceeds androsterone.

Case 13 is a man, aged 50; 17-ketosteroid output 20 mg. per day; dehydro*iso*androsterone 34 per cent of total ketosteroid.

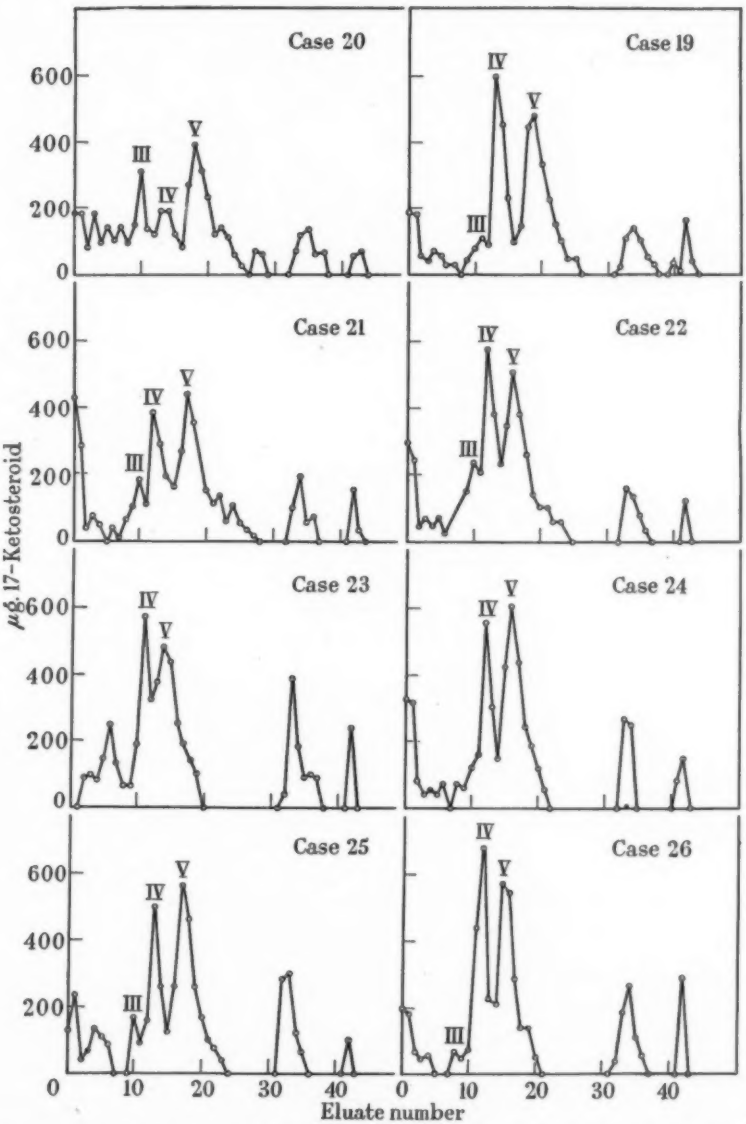

FIG. 2.—17-Ketosteroid content of eluates from chromatograms of urines from patients with prostatic disease.

Cases 19 and 20, prostatic hyperplasia.
Cases 21–26, carcinoma of the prostate.

TABLE I.—*Percentage Composition of 17-Ketosteriod Complex from Urines of Normal Males.*

Case No.	Age (years).	I.	II.	IIa.	III.	IV.	V.	VI.	VII.	VIII.
1	11	14·0	..	..	2·0	22·0	15·8	5·1	35·7	5·7
2	12	15·0	3·0	..	12·1	8·4	20·8	19·1	18·7	2·9
3	17	14·0	3·0	3·2	5·3	25·3	30·0	8·9	8·2	1·9
4	25	19·1	0·6	..	19·4	30·4	15·5	4·5	5·9	4·7
5	28	10·5	..	1·1	4·1	26·2	38·0	9·7	6·2	4·3
6	28	18·2	5·6	..	17·2	23·0	24·0	1·7	6·4	3·8
7	28	10·0	0·8	2·4	8·9	33·0	32·0	5·5	5·7	1·9
8	36	15·5	0·9	..	11·3	37·0	18·0	2·4	11·5	3·0
9	37	8·1	1·0	..	12·6	46·0	21·3	2·9	7·1	1·1
10	40	8·7	6·9	..	3·3	49·0	11·0	..	15·5	6·0
11	46	7·1	..	1·7	5·4	36·8	32·2	5·0	7·9	3·7
12	48	10·5	4·6	0·4	11·0	27·0	31·0	5·3	7·8	2·6
13	50	18·4	..	..	34·0	19·4	20·3	1·6	3·7	2·3
14	53	14·4	3·5	1·3	13·0	23·8	23·8	3·3	14·1	3·0
15	54	14·1	2·6	..	4·8	32·6	23·7	5·3	12·1	4·7
16	59	12·9	4·1	2·4	18·0	28·8	24·6	3·3	5·3	0·8
17	65	8·0	4·4	..	9·5	37·0	31·8	2·1	5·2	2·1
18	73	9·9	2·7	..	18·0	35·6	20·8	..	8·8	4·4

TABLE II.—*Percentage Composition of 17-Ketosteroid Complex in Males with Prostatic Disease.*

Case No.	I.	II.	IIa.	III.	IV.	V.	VI.	VII.	VIII.
19 (B)	8·6	4·6	..	5·9	28·9	36·9	1·7	9·2	4·0
20 (B)	9·4	5·8	8·1	12·0	12·7	28·3	10·2	10·6	3·0
21 (C)	15·2	4·6	..	10·0	21·5	29·6	8·1	8·4	2·9
22 (C)	11·3	4·1	..	16·3	22·8	33·1	2·5	7·8	2·1
23 (C)	2·1	15·9	..	5·1	19·0	33·6	4·4	15·1	4·6
24 (C)	13·1	3·7	..	5·1	23·4	37·0	3·2	9·9	4·6
25 (C)	8·0	9·9	..	5·5	21·3	34·1	3·8	15·4	1·7
26 (C)	9·0	1·8	..	4·0	26·4	35·9	3·6	13·4	5·9

(B) indicates benign enlargement of the prostate, (C) carcinoma of prostate.

of course, actually be present, since two or more 17-ketosteroids may be eluted together (this is known to occur in peak III, which may contain both dehydro-*iso*androsterone and *iso*androsterone). An examination of Tables I and II shows, however, that the major portion of the ketosteroids is accounted for in peaks III, IV and V, that is, those corresponding to 3-(β)-hydroxyketosteroids, androsterone, and aetiocholan-3-(α)-ol-17-one respectively. Further work may show that certain minor peaks, including those shown only by some urine extracts (e.g. IIa), may have even greater biological significance than the major ones, but the amounts isolated from these eluates have so far been too small for identification.

If attention is confined to the three major peaks III, IV and V, an examination of Table I shows no obvious trend in their relative amounts with age. Peak III, average value 11·7 per cent, ranges from 2 to 34 per cent of the total, peak IV averages 30 per cent, with a range from 8·4 to 49 per cent, and peak V has a mean of 24·2 per cent, with a range from 11 to 38 per cent. It will be seen that the average output of androsterone exceeds that of aetiocholanolone in the ratio 1·25:1·0. Only in three of the 18 cases shown in Table I does the output of aetiocholanolone notably exceed that of androsterone (Cases 2, 3 and 5); in the other 15 cases the amount of the former ketosteroid is either approximately equal to or less than the amount of androsterone.

A comparison of the data shown in Table II with those of Table I shows, however, what appears to be a significant change in pattern. In the urines from the cases of prostatic disease there is a consistent preponderance of peak v over peak iv. This is illustrated in Fig. 3, in which the relative amounts of the

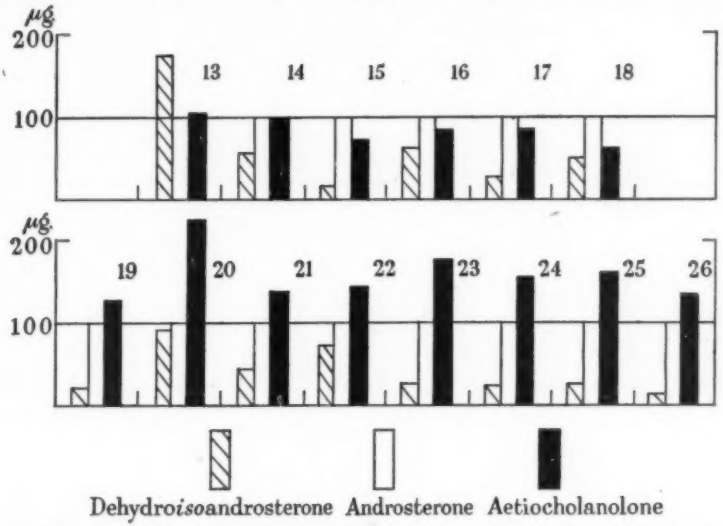

FIG. 3.—Relative excretions of dehydro*iso*androsterone, androsterone and aetiocholanolone in normal males and in males with prostatic disease.

Cases 13–18, normal.
Cases 19–20, prostatic hyperplasia.
Cases 21–26, prostatic carcinoma.

Amounts of dehydro*iso*androsterone and aetiocholanolone are shown relative to the same amount (100μg.) of androsterone in all cases.

three major peaks are shown for extracts prepared from urines of six normal males over 50 years of age (Cases 13–18), and from the urines of two subjects with prostatic hyperplasia (Cases 19 and 20), and six patients with carcinoma of the prostate (Cases 21–26). Urines from the carcinoma cases were obtained before oestrogen therapy was begun, and those from the benign hyperplasias before operation. For convenience of comparison, the amounts of peaks iii and v are shown relative to the same amount (100μg.) of peak iv (androsterone) in all cases. It will be seen that the most striking feature is the relative increase in peak v in the cases suffering from prostatic disease. In the normal males over 50 years of age the amount of this peak is equal to or less than the amount of androsterone, but in the 8 cases of prostatic disease it is present in considerable excess.

As mentioned earlier, the preliminary experiments with known mixtures of 17-ketosteroids showed that peak v normally indicates the presence of aetiocholanolone, and the total amount represented by this peak is a measure of the quantity of this steroid in the original mixture. It may be provisionally assumed, therefore, that the increased size of this peak in prostatic disease indicates a

relative increase in the output of aetiocholan-3-(α)-ol-17-one, although the possibility is not excluded that the increased peak size is caused by the inclusion of a second substance (of at present unknown nature) which is eluted with the aetiocholanolone. A decision on this question can be made only when sufficient of the material present in this peak has been accumulated to allow of its identification, and experiments are in progress with this object in view.

If it is assumed that aetiocholanolone is the substance that is increased in relative amount in prostatic disease, the cause of this increase is at the moment obscure. This ketosteroid has been recognized as a transformation product of more than one other steroid. Callow (1939) isolated aetiocholanolone and androsterone in approximately equal amounts from the urine of normal men, and also much larger, but still approximately equal, amounts from the urine of a man treated with testosterone. These two isomers appear to be end-products of testosterone metabolism, and to be formed in about equal amounts from this hormone. An explanation of increased testosterone metabolism does not appear to fit the present observations, however, since they show an increase in aetiocholanolone relative to androsterone. In this connection it may be remarked that Callow's finding of approximately equal amounts of these two ketosteroids in normal male urine was based on experiments with a pooled sample of urine, and an examination of Table I shows that in individual urines the ratio between the amounts of androsterone and aetiocholanolone is not constant, although the average values are approximately equal.

Aetiocholan-3-(α)-ol-17-one has also been shown to be a metabolite of dehydro-*iso*androsterone. Mason and Kepler (1945, 1947) found that administration of dehydro*iso*androsterone acetate by intramuscular injection in oil to patients with very low outputs of 17-ketosteroids (a man with anterior pituitary deficiency and a man and a woman with Addison's disease) led to greatly increased excretion of both aetiocholanolone and androsterone. In the two male subjects the amount of androsterone excreted exceeded that of aetiocholanolone by approximately 75 per cent, while in the female patient the aetiocholanolone was about 4·5 times as much as the androsterone.

The observed increase in the ratio of aetiocholanolone to androsterone in the cases of prostatic disease might be produced by an increase in the absolute amount of aetiocholanolone, or by a decrease in the absolute amount of androsterone excreted, or by both simultaneously. The amounts of 3-(β)-hydroxyketosteroids, androsterone and aetiocholanolone excreted in 24-hours were calculated for the cases illustrated in Fig. 3. The results showed that the average daily excretion of aetiocholanolone in the two groups of cases (normal and prostatic disease) were approximately equal (2·0 and 1·9 mg. per day respectively), but that the androsterone excretion in the normal group (2·2 mg. per day) exceeded that of the prostatic group (1·25 mg. per day). These figures appear to support the conclusion that the actual difference between the two groups of subjects consists in a diminished output of androsterone by the prostatic group. This conclusion must, however, be regarded as provisional. Calculation of the daily excretions corresponding to peaks III, IV and V for all the cases shown in Table I indicates that there is a gradual decline with advancing age of each of these groups of substances, and hence the absolute excretions shown by the prostatic group (average age 65 years) should more properly be compared with a group of normal men of the same average age rather than with the slightly younger group (average

age 59 years) shown in Fig. 3. Such a comparison will be possible when further normal males in the later decades have been examined.

In a study of α-ketosteroid excretion in patients with neoplastic disease Dobriner, Rhoads, Lieberman, Hill and Fieser (1944) found abnormal patterns in several cancerous conditions. In the one case of prostatic cancer reported in their paper there was a great excess of aetiocholanolone as compared with androsterone. They found a similar relation in a case of carcinoma of the larynx and a case of carcinoma of the breast. From the present results it appears, however, that such a relation is not necessarily characteristic of neoplastic disease, since the two cases of benign hyperplasia (Cases 19 and 20) show the same excess of aetiocholanolone; in one of them, indeed (Case 20), aetiocholanolone is present in greater excess than in any of the neoplastic cases.

Whatever the ultimate explanation may be of the relative increase of aetiocholanolone, it will be of great interest to investigate the effect of oestrogen therapy on the urinary 17-ketosteroid pattern in these cases of prostatic carcinoma. Material has been collected for this purpose and is being examined.

SUMMARY.

Methods for the chromatographic separation and estimation of 17-ketosteroids in urine extracts are discussed, and a standard technique which yields reproducible analyses is described.

Urines from 19 normal males ranging in age from 3 to 73 years and urines from 2 cases of benign hyperplasia of the prostate and from 6 cases of carcinoma of this gland were examined by this method.

In normal males the amounts of androsterone and of aetiocholan-3-(α)-ol-17-one are approximately equal, but in prostatic disease the latter substance is present in relative excess.

The significance of this observation is discussed.

Generous grants to this Hospital by the British Empire Cancer Campaign have supported this work. We should also like to thank the Medical and Nursing Staff of St. Bartholomew's Hospital for helpful co-operation.

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THE CULTIVATION OF TUMOURS IN THE FERTILE EGG, WITH SPECIAL REFERENCE TO ASSOCIATED ECTODERMAL LESIONS OF THE CHORIOALLANTOIC MEMBRANE.

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THE original purpose of this investigation was to study the effects of implanting various tumours affecting the fowl into the fertile egg.

A survey of the literature revealed the rather surprising fact that apart from the Rous I sarcoma (Rous and Murphy, 1911), and a few leucosis cases (Pierce, 1942), no attempt has been made to grow spontaneous neoplasms of the chicken in fertile eggs.

There is a high incidence, relative to disease in general, of spontaneous neoplasia in the fowl. Campbell (1945), in a survey extending over five years, found the relative average incidence to be 18·7 per cent in the breeds examined, the minimum and maximum figures for specific breeds being 10·3 per cent and nearly 39 per cent.

During this survey a number of chicken tumours were studied which bore a close morphological resemblance to known virus-associated growths. In view of claims by various authors (Taylor, 1943 ; Heilman and Bittner, 1944 ; Hungate, Snider, Taylor and Thompson, 1945) that mammalian tumours can be easily cultivated in the yolk-sac of the chick embryo, and that a virus-like factor associated with mammary carcinoma of mice has been directly demonstrated by this method, it was thought desirable to apply similar methods to the study of spontaneous chicken tumours.

The advantages of using fertile eggs for such cultivation experiments are many. The egg with the developing embryo represents an enclosed stable biological system in which implanted tumours or inoculated viruses can grow under optimum conditions of sterility, controlled temperature and nutritive environment. Also the embryonic membranes quickly provide a vascular bed from which tumour cells can obtain their blood supply. It was argued that if mammalian tumours grow so well in the chick embryo, then tumours derived from autologous tissues should grow at least as well, or perhaps even more readily.

One of the disadvantages associated with laboratory animals used for tumour transplantations, etc., is a natural immunity due to the presence of antibody, either resulting from sub-clinical infection, or produced by the animal in response to inoculation with a virus-associated tumour. Also Carr (1943) has shown an immunity to the Rous I sarcoma in a certain strain of chickens which is apparently not due to serum antibodies. Apart from the fact that any laboratory animal can only be used for experiments with tumours of that particular species (with

* Part of this work was done while the writer was on the staff of the Royal (Dick) Veterinary College, Edinburgh.

the exception of the anterior chamber of the eye method), some of them, e.g. mice, have been shown to be naturally infected with any one of at least six different viruses, and so lead to confusion in passage experiments. The chick embryo, on the other hand, has not yet been reported to be a natural carrier of virus.

It has been shown by Grasset (1929), who worked with diphtheria and tetanus toxoids as antigens, that the chick embryo does not produce antibody. Polk, Buddingh and Goodpasture (1938) showed that no complement is present in embryonic serum, and Murphy (1914) first demonstrated the ability of normal heteroplastic tissues and tumours to graft on to the chorioallantois without causing an inflammatory reaction until the 18th day of incubation. Subsequently some reaction of the host tissues does occur, to the detriment of the graft.

The culture of tumour tissues in fertile eggs therefore resembles much more closely tissue culture *in vitro* than it does in orthodox animal inoculation methods.

At an early stage in this investigation the frequent occurrence of typical ectodermal lesions in the vicinity of chicken tumours implanted on the chorioallantois led to the conception that such tumours might contain a factor capable of stimulating cell division. Such a factor, if it existed, might be a self-propagating agent within living malignant cells, or something in the nature of a mitotic activator similar to those growth-stimulating substances present in embryonic tissues.

With this hypothesis in mind it was decided to study the effects of implanting embryonic tissues, certain viruses, and as wide a range of tumours as possible, derived from a variety of animals.

About this time a series of cases of spontaneous liver carcinoma in ducks came under observation, and similar experiments were undertaken with the resultant material, the details of which are recorded in a separate paper.

The first record of tumour cultivation in the fertile egg was by Rous and Murphy (1911), who applied it to the newly discovered Rous chicken sarcoma, which they succeeded in cultivating in various sites, including the yolk-sac, and on the chorioallantoic membrane. They found that even the dried and powdered tumour gave rise to tumours upon inoculation into the fertile egg. Growth took place mainly by amitotic division. They noticed an increased embryo mortality on successive implants, and failed to carry their transplants beyond the 4th generation. They make no mention of any reaction on the part of the chick embryo or its associated membranes, and their plates only illustrate the histology of the cultivated tumours; the membranes themselves were apparently not examined.

Stevenson (1918) then tried various rat, mouse and guinea-pig tumours and had about 30 per cent takes. No mention was made of reactive processes or lesions developing in the chorioallantois adjacent to the tumours. Similarly Minoura (1921), in a study of testis and ovary grafts in the hen's egg, only mentioned an abnormal accumulation of leucocytes in the region of implantation. Huxley and Murray (1924) implanted chick embryo fragments on the chorioallantois. They reported a general thickening of the mesenchyme, proliferation of capillaries and cornification of the ectoderm, which may form a stratified epithelium. They also noted the formation of epithelial "pearls," and remarked on the fact that these were distinctly localized, "pearls" being absent from one part of reaction thickening, and abundant in another part.

Keogh (1938) found that filtrates prepared from the Rous I sarcoma gave rise to ectodermal proliferative lesions when inoculated on the chorioallantois.

These focal lesions were fully developed about the 7th day after inoculation, and took the form of flattened pearly opacities varying in diameter from 0·5 mm. to 2 mm. Dilute inocula produced these lesions ; when concentrated suspensions were inoculated a proportion of sarcomatous lesions developed.

Pierce (1942) transferred leucosis to chick embryos by seeding fragments of leucotic tissue, e.g. spleen, on to the chorioallantoic membrane. The leucosis was on one or two occasions retransmitted to chicks by inoculation with filtrates obtained from membranes which had previously been inoculated with Berkefeld filtrates from leucotic material. Most of the experiments of this nature, however, gave negative results. No lesions developed on the membrane, and inclusion bodies were absent when filtrates were used, but leucotic tissue caused gross thickening and cloudiness of the membrane.

Taylor, Thacker and Pennington (1942) described the growth of cancer tissue (mammary carcinoma of mice) in the yolk-sac of the chick embryo, and claimed it to be a new method. They injected 0·25 ml. of tumour suspension into fertile eggs of 4–5 days' incubation. After incubation at 37°C. for 12–13 days, tumours were found growing into the yolk cavity from the yolk-sac wall. They claimed 100 per cent takes, and transmitted the tumours back to mice. The presence of necrotic tissue in the tumours resulted in the death of the embryo.

In a subsequent communication Taylor (1943) described the cultivation of a spontaneous mammary tumour of dba strain mice in the yolk-sac of the fertile egg. The yolk from such eggs produced similar tumours in mice upon injection, and Taylor found that Berkefeld filtrates prepared from this yolk also gave rise to tumours upon injection into mice, thus showing the presence of a virus-like principle in this particular mammary carcinoma.

At about the same time Heilman and Bittner (1944) injected a 40 per cent suspension of mouse carcinoma into the yolk-sac of the embryonic chick, using an inoculum of 0·2 ml. They extracted the tumours from the yolk-sac, and showed that filtrates prepared from these and from the yolk itself were capable of causing mammary cancer in mice, thus confirming the work of Hungate, Taylor and Thompson (1944), that a virus-like body was involved. Following up this line of investigation Bittner, Evans and Green (1945) showed that the " milk factor " was able to survive in the yolk-sac for 12 days in the absence of mouse mammary carcinoma cells. They did not know whether the factor had multiplied in the yolk-sac.

Twombly and Meisel (1946) attempted to grow several mammalian tumours in the yolk-sacs of fertile eggs. The greatest number of takes occurred with the Bagg 755 mouse mammary carcinoma, but they report a very high mortality rate (73 per cent) by the 17th day of incubation in their embryos. Numerous filtration experiments were done in an attempt to demonstrate a cancer virus, but with uniformly negative results.

METHODS.

(a) *The intra-vitelline technique.*

The method adopted for the cultivation of tumours in the yolk-sac was a modification of that described by Taylor *et al.* (1942) and Hungate *et al.*

(1944). Fertile hen eggs at the 4th–5th day of incubation, supplied by Dr. A. W. Greenwood from his strain of pedigree Brown Leghorns, were used throughout the investigation.

During injection the egg was held on a special stand as described by Beveridge and Burnet (1946), and the whole operation was performed under a glass canopy open at the front, formed by placing a large accumulator jar on its side. All instruments and materials were kept under this canopy during the actual inoculations. At the termination of the experiment sterile broths were inoculated with a few drops of the tumour suspension still within the syringe, and incubated for 24 hours as a check on the sterility.

All injected eggs were incubated for a period not exceeding 14 days. In those cases where candling showed the deathof the embryo s a bacteriological examination of the egg contents was always made.

(b) *The chorioallantoic technique.*

The membrane was exposed for implantation by the method described by Beveridge and Burnet (1946), using a dental drill (Alpine no. A.137) operated through a flexible shaft and powered by an electric motor with foot control. Tumour fragments were stored in saline and implanted by means of a pipette on to the membrane. In most cases the shell flap was replaced and sealed with paraffin wax, but occasionally a cover-slip was substituted to serve as a window, and allow the implanted tumour to be examined for size, vascularity, haemorrhagic change, etc.

(c) *Direct homologous or heterologous inoculation.*

The techniques were simply those of subcutaneous, intramuscular, intravenous or intra-peritoneal injection, and need not be described further.

(d) *Histological methods.*

Egg membranes with implanted tumours were trimmed to a suitable size and shape with fine-pointed scissors and transferred immediately to Susa's fixative for 1 hour. They were then taken direct to 95 per cent alcohol, through absolute alcohol, equal parts absolute alcohol and cedarwood oil, cedarwood oil alone, and finally embedded in paraffin wax.

Other tissues were fixed in 10 per cent formol saline for 24 hours. They were then transferred to phenol-methylated spirit solution (5 per cent) through absolute alcohol, then equal parts alcohol and benzol, benzol alone, equal parts benzol and paraffin wax, and finally embedded.

Sections were cut at approximately 7μ, and were stained with Ehrlich's acid haematoxylin and eosin, also by Mallory's method, and with Wilder's silver impregnation technique to demonstrate reticulum. Many of the tumour-bearing membranes were studied as serial sections.

EXPERIMENTS AND RESULTS.

(a) *Virus-associated Tumours.*

Experiments 1, 2 and 5 were undertaken in order to study the growth of a known virus-associated tumour in the fertile egg, and also to ascertain the nature

of the lesions, if any, which developed in the chorioallantois in the immediate vicinity of the implanted tumour.

Experiments 1 *and* 2.—Seven 10- to 11-day embryonated eggs were implanted with small fragments of a Rous I sarcoma growing in the pectoral muscle of a brown Leghorn chicken. The eggs were sealed with their own shell flap, using paraffin wax, and this was removed on the fifth day and a cover-glass substituted. Tumours were seen growing in each, dependent from the chorioallantois. On opening the eggs on the 18th day of incubation the largest tumour measured 15 × 10 × 8 mm., and was surrounded by several smaller growths in a thickened opaque membrane. In one instance the central region of the tumour was haemorrhagic, and it was usual to find numerous opacities and thickening of the membrane in the region of the sarcoma (Fig. 1).

One of these tumours was ground in saline and a cell-free extract injected into a young Brown Leghorn chicken. A typical Rous I sarcoma appeared within three weeks at the site of injection.

Experiment 3.—An attempt to subcultivate the egg-grown tumours on the chorioallantois was not successful. A Rous I sarcoma grown for seven days on the embryonic membrane was removed, minced with scissors, and fragments implanted into three 7-day embryonated eggs. The embryos died in two days, and cultures resulted in the isolation in a pure form of *Staphylococcus aureus*.

Experiment 4.—The reaction of the chorioallantois to a cell-free filtrate of Rous I sarcoma was studied as follows:

Four 12-day embryonated eggs each received 0·02 ml. filtrate from a Rous I sarcoma growing in a chicken. Two eggs were examined four days later, but to the naked eye no lesions were discernible. The remaining eggs were opened

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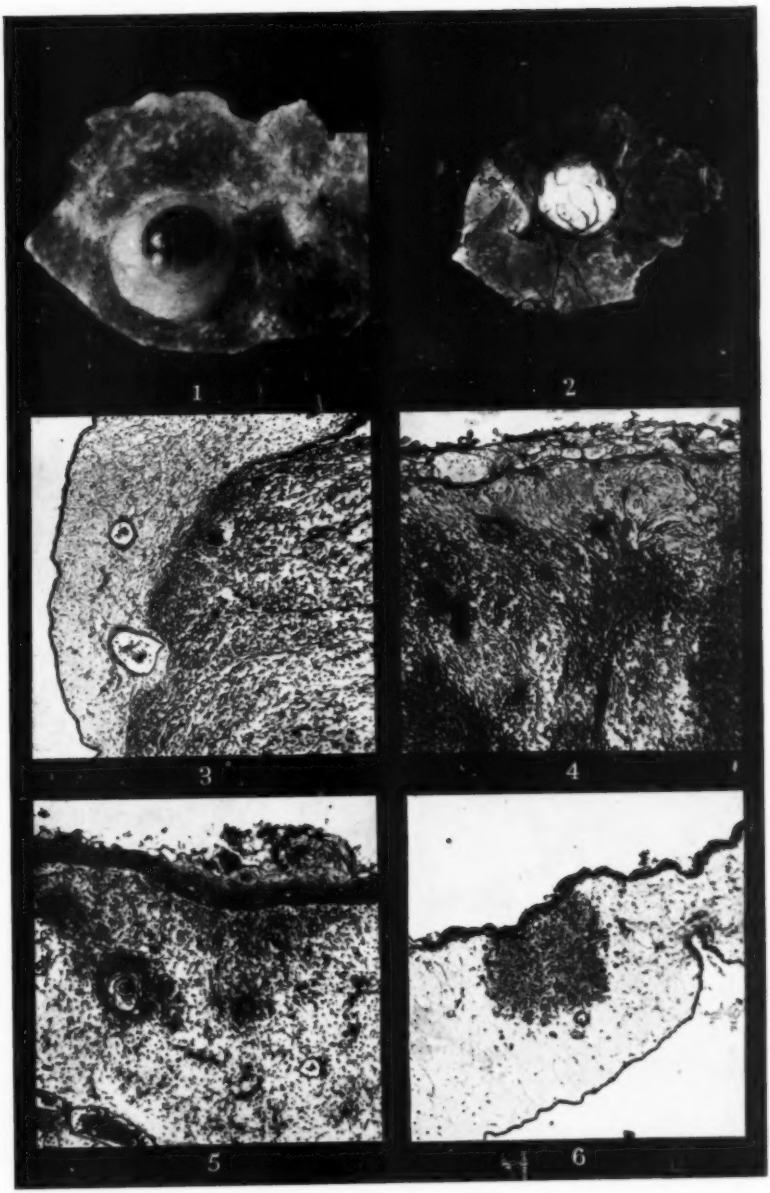
EXPLANATION OF PLATES.

FIG. 1.—Rous I sarcoma, cultivated on the chorioallantois of the chick embryo. × 1⅔.
FIG. 2.—Duran-Reynals sarcoma, cultivated on the chorioallantois. × 1.
FIG. 3.—Histology of Rous I sarcoma in the chorioallantois. H. & E. × 58.
FIG. 4.—Histology of Duran-Reynals tumour in the chorioallantois. H. & E. × 58.
FIG. 5.—"Cell-nest" and ectodermal proliferation and keratinization adjacent to Rous I sarcoma in the chorioallantois. H. & E. × 58.
FIG. 6.—Proliferating and infiltrating ectodermal cells of the chorioallantois subsequent to inoculation with Rous I virus. H. & E. × 58.
FIG. 7.—Swollen actively dividing ectodermal cells of the choricallantois due to Rous I virus. Note inclusion-like body. Mallory. × 700.
FIG. 8.—Inclusion bodies of fowl pox virus in swollen proliferating ectodermal cells of chorioallantois, for comparison with previous figure. H. & E. × 540.
FIG. 9.—Methylcholanthrene induced mammary carcinoma of mouse growing in yolk-sac of chick embryo. × 3·3.
FIG. 10.—Ectodermal proliferation and "cell-nests" adjacent to a methylcholanthrene-induced mammary carcinoma of mouse, in the chorioallantois. H. & E. × 58.
FIG. 11.—G.R.C.H./15 (1.2.5.6 dibenzanthracene-induced fibro-sarcoma of chicken) growing on the chorioallantois. × 1.
FIG. 12.—Histology of G.R.C.H./15 in the chorioallantois. H. & E. × 58.
FIG. 13.—Haemocytoblasts in liver of chick embryo. H. & E. × 265.
FIG. 14.—Myelcid leukaemia. Heart blood, chick embryo. Leishman. × 550.
FIG. 15.—Embryonal nephroma. Light Sussex hen. × 8.
FIG. 16.—Embryonal nephroma growing in chorioallantois. × 1⅔.
FIG. 17.—Ciliated and mucus-secreting cells resembling bronchial epithelium, in the original nephroma. H. & E. × 265.
FIG. 18.—Embryonal nephroma growing in the chorioallantois, consisting only of embryonic (mesonephric-like) tubules and stroma. Note "cell-nests" adjacent to implant. H. & E. × 58.



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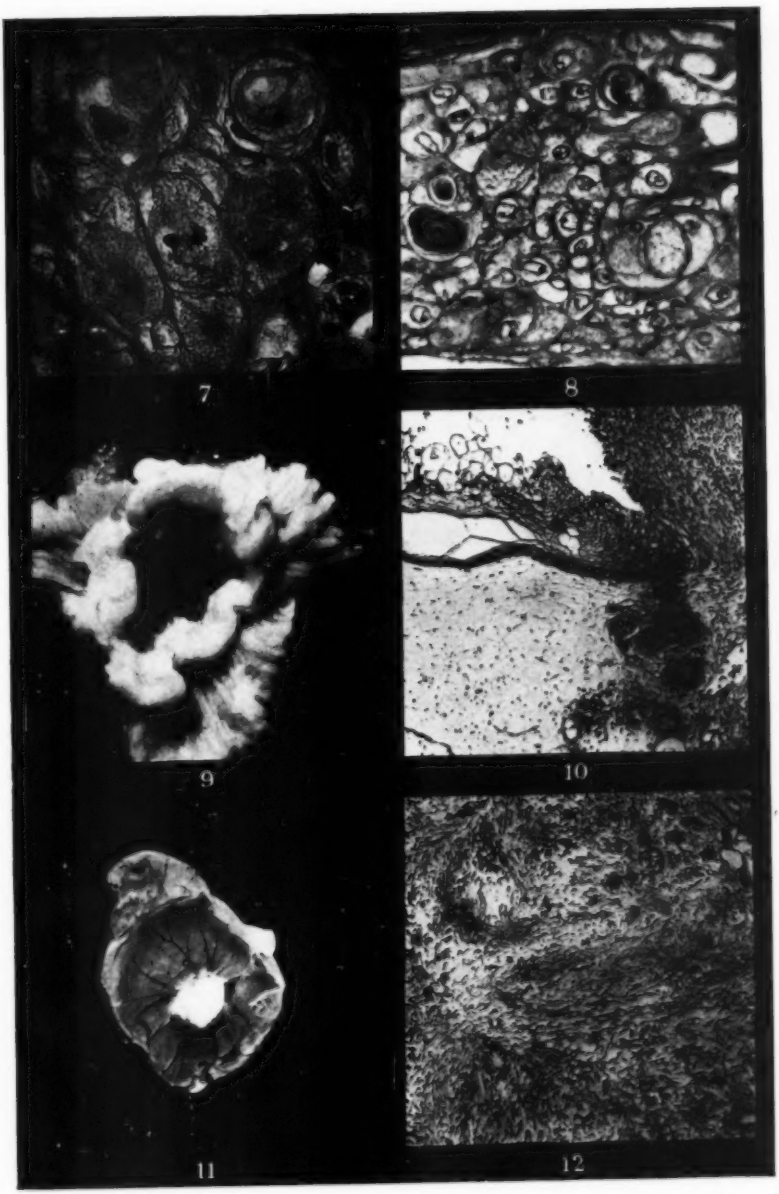

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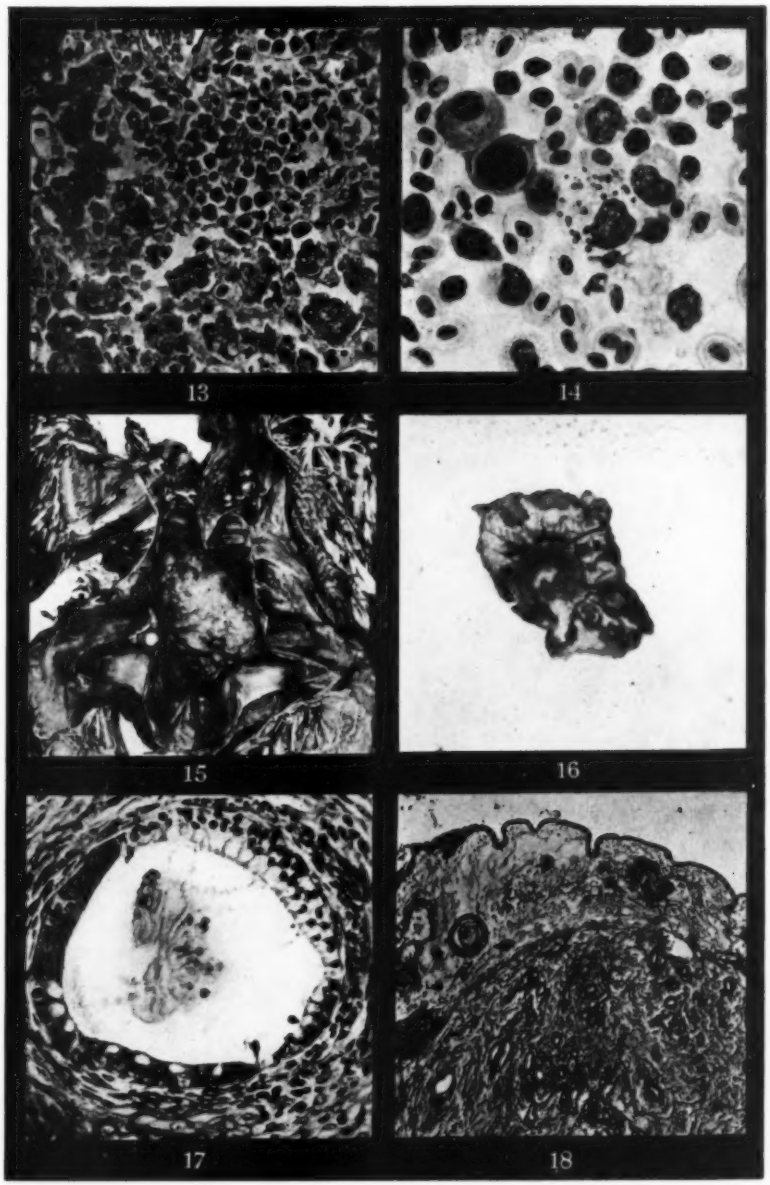

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on the 6th day and the membranes were seen to be slightly thickened, with one showing many small discrete opaque foci.

Experiment 5.—Two 10-day embryos were implanted with fragments of a Duran-Reynals type "D" sarcoma from a Brown Leghorn cockerel. This tumour is similar to the Rous I series, but is said to give rise to haemorrhagic lesions more frequently in the host. The eggs were opened 9 days later, and both contained large (9 mm.) spherical, richly vascular tumours growing from the chorioallantois and hanging down into the allantoic cavity. The surrounding membrane was thickened and (Fig. 2) showed small focal opacities and haemorrhages. One tumour showed haemorrhages. A tumour and membrane were kept for histological examination, whilst the other was minced and a filtrate prepared from it. 0·5 ml. of this filtrate was injected into each pectoral muscle of a Brown Leghorn cockerel aged 14 weeks. Typical tumours subsequently grew in these sites.

Histology.

The only histological findings to be given in any detail will be those associated with changes in the membranes in the vicinity of implanted tumours. The tumours themselves will only be described in the briefest terms, except where notable differences occur between the primary and the cultivated tumour.

Both Rous and Duran-Reynals sarcoma grow well on the chorioallantois (Fig. 3 and 4). Although implanted on the epithelial surface, the main body of the tumour grows downwards, so that the base is epithelial, whilst the greater curvature is invested with endothelium. The sarcoma cells occupy the mesoderm, which they actively infiltrate, and apparently stimulate the connective-tissue cells of the chorioallantoic membrane to participate in the neoplastic progress. The tumours are richly vascular, and frequently exhibit haemorrhagic or necrotic areas. A delicate argyrophil reticulum ramifies throughout the growth, and appears to be derived from the mesenchyme of the membrane.

Normally the epithelial surface of the chorioallantois consists of low cuboidal cells, and is only one cell thick. Under the influence of the Rous and Duran-Reynals tumours it proliferates rapidly and becomes many cells deep (Fig. 5). The cells in the deepest layer are polyhedral, basophilic, and contain large vesicular nuclei, with a prominent nucleolus. They tend to grow down as finger-like processes into the mesenchyme, and even exhibit infiltrative properties (Fig. 6). The more superficial cells are eosinophilic, and either become flattened or grossly enlarged to form a spherical cell exhibiting distinct cell walls surrounding a fine granular eosinophilic cytoplasm and a pale staining vesicular nucleus. Mitotic figures are numerous in all these cell types, and vesicular inclusions are occasionally seen (Fig. 7). The most superficial layer consists of flattened keratinized cells, and large swollen vacuolated cells with pyknotic nuclei. Such cells frequently contain small brilliantly eosinophilic granular aggregations, the nature of which is obscure.

Many of the ectodermal down-growths appear in section as typical "epithelial pearls," or "cell nests." They have an outer layer of basophilic low-cuboidal epithelium corresponding to the deepest layer of the mass of proliferating ectodermal cells of the chorioallantois. Mitotic figures are frequent. Passing towards the centre of the "cell nest," the cells become progressively flattened

and keratinized, and the central region is occupied by a structureless, intensely eosinophilic mass of hyaline material.

(b) *Action of Dermatrophic Virus on the Chorioallantoic Membrane.*

In order to have a basis of comparison for the ectodermal lesions produced by the Rous and Duran-Reynals virus, eggs were inoculated with fowl pox virus, known to produce lesions and typical inclusion bodies in the ectoderm (Woodruff and Goodpasture, 1931), and also with the virus of contagious papilloma (" angleberries ") of cattle.

Experiment 6.—Fowl pox virus was prepared as follows: 0·5 g. of dried vaccine was placed in a sterile tube containing glass beads and 10 ml. normal saline, and the tube was closed with a boiled rubber bung. The tube was shaken in an automatic shaking machine for 1 hour, at the end of which the suspension was filtered through a " Technico " filter, using a Ford's " Sterimat " Grade G.S. The filtrate was used as the inoculum, the amount inoculated on to each membrane varying between 0·02 to 0·06 ml.

Four 10-day embryonated eggs were inoculated, using a graduated capillary pipette as a dropper. In order to secure a wide distribution of the virus over the membrane, it was placed direct on to the shell membrane, and a tear made through this with a glass needle. The virus was then drawn into the egg and on to the chorioallantois by applying negative pressure to the air-cell with the aid of a small rubber teat. The egg was then sealed in the normal way and incubated for 3 days. When opened and examined, small roughly circular opacities were found on the chorioallantois of 3 embryos. The fourth was found to be dead and infected, probably due to accidental cracking of the shell during drilling.

Histology of lesion.

The mesenchymal tissue is thickened due to connective-tissue proliferation, oedema, and a fairly intense infiltration of granulocytes. Ectodermal proliferation is marked. The cells are large with vesicular nuclei, and are spherical or polygonal, with a well-defined outline. Definite eosinophilic virus inclusion bodies occur in the cytoplasm (Bollinger bodies) (Fig. 8).

Within the mesenchyme of the chorioallantois islands of epithelial cells occur, also showing inclusions. An occasional attempt at the formation of a " cell nest " may be found, but usually the cells occur in groups without any sign of a whorled appearance. The endothelial aspect of the chorioallantois is much folded in these regions of thickening, but there is no sign of endothelial proliferation.

Experiment 7.—Fresh papillomata from a natural case in a bovine were ground up with sand and saline, and the suspension filtered as before to obtain the virus. This was inoculated in the usual way on to the chorioallantois of one 8-day embryo using a dose of 0·1 ml. The egg was opened on the 18th day of incubation and the membrane cut out and examined in saline. No opacities were seen, but the membrane appeared to be a little less flexuous than normal.

Histologically the membrane was thickened, due to proliferation of the mesenchymal tissue. Minute ectodermal proliferations were present, but no definite inclusion bodies were detected, neither were any " cell nests " present.

(c) *Chemically Induced Tumours (Virus free?)*

Four experiments were performed using chemically induced tumour tissue from the mouse. Two of these experiments were yolk-sac cultivation attempts, the tumours used being methylcholanthrene induced transplantable mammary carcinoma, and the first attempt (Experiment 8) was a complete failure, owing to necrotic changes in the original tumour.

Experiment 9 was a repeat of the above, and of the 13 eggs injected on the 4th day of incubation, all embryos died at various stages save one, which when examined on the 18th day of incubation showed a ring of white tumour tissue surrounding the yolk-sac umbilicus (Fig. 9). The histology of this growth was very similar to that of the original tumour, i.e. carcinoma simplex, with large round and spindle-shaped cells.

Experiments 10 and 11 were chorioallantoic implants using methylcholanthrene induced transplantable mammary carcinomas of mice. Five 10-day embryonated eggs were implanted with fragments of tumour and examined 7 days later. All but one contained small tumours 3–5 mm. diameter. The membranes adjacent to these growths were thickened and somewhat opaque. A definite zone of opacity surrounded all the tumours.

Histological examination of the tumours shows typical carcinoma simplex, but with a good deal of connective-tissue stroma and some areas of necrosis. The surrounding chorioallantois is thickened, mainly due to oedema, and the epithelium shows advanced proliferative changes as previously described, with abundant formation of "cell nests." The epithelial cells of the outermost layer are swollen and cystic, being devoid of cytoplasm and nucleus and leaving only a keratinized cell-border (Fig. 10).

Experiments 12 and 13 with rat tumours (Walker rat carcinoma) resulted in 100 per cent mortality in the 20 embryos receiving implants. Subsequent histological examination showed that the original tumours contained large areas of necrosis.

Experiment 14.—Two 10-day embryonated eggs were implanted with a chemically (1:2:5:6-dibenzanthracene) induced fibro-sarcoma of the fowl. The tumour, one of a series so produced by Dr. Peacock of the Glasgow Royal Cancer Hospital, is known as G.R.C.H./15. All attempts to demonstrate a virus in this sarcoma have up to the present been unsuccessful (Peacock, 1946).

The eggs were opened on the 9th and 10th days, and both contained well defined spherical tumours of 5 mm. and 8 mm. diameters respectively. There was no sign of reaction in the membranes surrounding the tumours (Fig. 11).

Histologically G.R.C.H./15 is a dense, vascular fibro-sarcoma, exhibiting many mitotic figures. Cultivated on the membrane the tumour is essentially similar to the original. Some areas are more loosely cellular (Fig. 12) and there is a very rich vascularity, also areas of haemorrhage and necrosis. The ectoderm of the chorioallantois adjacent to the tumour is practically normal. That investing the tumour, however, exhibits proliferation and swollen empty keratinized cells. "Cell nests" are absent from this region, but attempts at their formation may be seen in the body of the tumour by down-growth in the usual way, and also by proliferation of displaced and engulfed epithelial cells by the rapidly growing tumour implants.

(d) *Spontaneous Mouse Tumours.*

Experiments 15 *and* 16.—Twenty-eight eggs incubated between 4 to 5 days had injected into the yolk-sac 0·25 ml. of a suspension of mouse sarcoma S.37 in normal saline. Twenty-seven embryos subsequently died at periods varying between 1 and 8 days. Most of these showed congestion and petechial haemorrhages in the skin. No bacteria were isolated from these eggs. One had a small tumour growing in the yolk-sac, attached near the umbilicus. The one surviving chick was removed from the egg on the 19th day, and showed small tumours in the yolk-sac.

S.37 mouse sarcoma is a polymorphous cell sarcoma. The two tumour-bearing eggs showed some diversity of structure in the growths. There were round cell areas, spindle areas, and areas showing both these types of cell. Unfortunately it was not possible to repeat these experiments using the chorioallantoic technique owing to the failure to obtain more S.37 mice. There were six saline injected eggs to serve as controls in these experiments, and they all hatched normally.

Experiment 17.—0·25 ml. of a suspension of a transplantable mouse lymphosarcoma were injected into the yolk-sacs of each of nineteen 4–5-day embryonated eggs. All the embryos died at varying periods up to the 11th day following the injection. They were bacteriologically sterile. The dead embryos showed intense congestion and petechial haemorrhages. In 2 cases tumours were found attached to the interior of the yolk-sac. Tumour-bearing membranes were ground up with sand and saline, and 0·25 ml. of the resultant emulsion injected subcutaneously into 10 mice. A haemorrhagic membrane was treated in the same way and injected into 5 mice. Nine days later the mice were found dead, due to a heating failure during a cold spell. Six of the mice injected with tumour-bearing membranes were found to have small lymphoid tumours growing in the subcutis at the site of injection. As with the S.37 experiments, it was not possible to repeat these observations using the chorioallantoic method of cultivation, because no more tumour-bearing mice were available.

Experiment 18.—Three 8-day embryonated eggs received implants of fragments obtained at biopsy of an osteogenic sarcoma from the hind leg of a dog. Control nutient broths inoculated with material from this tumour remained sterile.

Nine days later the eggs were opened and no trace of the implants could be found. The membranes appeared normal upon histological examination.

(e) *Spontaneous Chicken Tumours.*

In Experiments 19, 20 and 21 the intra-vitelline method was again tried. These experiments may be briefly dismissed, as in each case contamination with bacteria killed all the eggs. The material for Experiment 19 was a primary liver carcinoma of a chicken. This was injected in the usual manner into 24 4-day embryonated eggs, all of which died within 6 days. *Bact. coli* was isolated from the eggs, although broths inoculated with tumour tissue from the original case remained sterile.

Experiments 20 and 21 consisted of the injection of a saline suspension prepared from lymphocytomata, involving the thigh and ovary respectively. All the embryos died subsequent to injection, and *Bact. coli* and a streptococcus were isolated from the eggs. Material from the thigh lymphocytoma was also

injected intramuscularly into a Brown Leghorn chicken with negative results when the bird was destroyed 13 months later.

The next ten experiments consisted of chorioallantoic implants.

Experiment 22.—Two 6 to 7-day embryonated eggs were implanted with fragments of an ovarian adeno-carcinoma from a Brown Leghorn hen which had died a few hours previously. Both embryos died within 3 days, and the original material and the eggs were found to be infected with *Bact. coli*.

Experiment 23.—Six 7-day embryonated eggs received implants from an ovarian adeno-carcinoma from a Brown Leghorn hen. This tumour had spread in the abdominal cavity by transcoelomic implantation to the mesentery and serosa of the gut and oviduct. Tumour fragments were obtained from the two latter sites. The eggs were opened on the 18th day of incubation, and only one bore a small tumour (3 × 2 mm.) on the membrane. This was not much bigger than the original implant. Histologically this proved to be necrotic. There was no reaction in the membrane adjacent to the implant.

Experiment 24.—Ten 9-day embryonated eggs were implanted with fragments of an ovarian adeno-carcinoma from a Black Leghorn hen. Broths subsequently inoculated with material from this tumour showed contamination with *Bact. coli*. Three embryos died within 2 days of implantation. Of the remainder, 6 developed tumours. These were spherical, yellowish in colour, and measuring about 5 mm. in diameter. An opaque zone of reaction was present in the membranes surrounding the tumours.

Histologically the tumour implants showed a great deal of necrosis. The plain muscle and fibrous tissue moiety had persisted longer than the epithelial elements. The adjacent membrane showed epithelial proliferation, with irregularly shaped small eosinophilic inclusions in the swollen cells. There were no "cell nests." The mesenchyme of the membrane was heavily infiltrated with granular leucocytes.

One of these tumours was re-implanted on to the chorioallantois of a 9-day embryo, with the results given below.

Experiment 25.—This died on the 5th day, due to streptococcal infection. The graft was found to be necrotic, and there was no membrane reaction in its vicinity.

Experiment 26.—Fragments of a friable ovarian adeno-carcinoma from a cross-bred hen were implanted on the membranes of four 10-day embryonated eggs. Broths inoculated at the same time with tumour tissue showed a streptococcus in pure culture on the following day. Examination of the eggs by transillumination, however, showed the embryos to be alive. They were examined on the eighth day, and three were found to contain tumours on the chorioallantois. These varied between 2 and 3·5 mm. in diameter. There were no detectable lesions on the membrane.

The original growth had the appearance of a granulosa-cell tumour exhibiting a "cylindroid" structure. On the membrane this is not so apparent, mainly owing to the growth of the epithelial cells at the expense of the tumour trabeculae of connective tissue, and to a gross infiltration of eosinophilic granular leucocytes. There is some proliferation of the ectodermal cells of the membrane and a few "cell nests" are present.

Experiment 27.—Two of the tumours grown on the chorioallantois in the previous experiment were re-implanted into two 8-day embryonated eggs. These were opened 10 days later and both contained small yellowish growths on the

membrane, surrounded by many minute irregular opacities extending for 7–8 mm. each side of the implant.

Histological examination of one membrane shows a few islands of surviving tumour cells, general thickening, ectodermal proliferation, and the presence of large numbers of well-formed " cell nests."

The remaining membrane was ground with sterile sand and saline, and the resulting suspension injected intraperitoneally into two 6-week-old Brown Leghorn chickens (females) with negative results when the birds were destroyed 18 months later.

Experiment 28.—Three 9-day-old embryos received implants on to the membranes with fragments of both liver and spleen from a R.I.R. hen suffering from aleukaemic lymphoid leucosis. Eight days later two of the embryos were found to be dead, namely those with liver and liver plus spleen implants. Only the latter had a tumour in the membrane. The remaining spleen only implanted embryo was living, and the membrane bore a 4 mm. diameter pink tumour. In the case of the dead embryos both were extremely congested, and the livers appeared swollen and leukaemic.

Histological examination of the membrane bearing the spleen implant shows a vascular growth of endothelial-like structure. All resemblance to normal or even leukaemic spleen has disappeared. The growth is composed of an irregular syncytium of cells with round, angular or elongated nuclei, the cytoplasm of which is drawn out into processes which appear to merge with those of adjacent cells. Rounded deeply staining cells (haemocytoblasts ?) lie in the interstices of this syncytial stroma. The vessels are formed of a single layer of flattened endothelium and are congested. At the periphery of this tumour large numbers of primitive blood cells appear to be wandering into the mesenchymal stroma of the chorioallantois. " Cell nests " occur in this region. One or two small areas of necrosis in the centre of the tumour are surrounded by giant cells containing many nuclei. " Cell nests " arising from ectodermal proliferation are present in the membrane adjacent to the implant.

The liver plus spleen implant shows an essentially similar picture to the above, as the liver moiety has disappeared. In its place is a dense scar of connective tissue and collagen, infiltrated with various types of wandering cells. The adjacent membrane shows proliferation of ectoderm and the formation of " cell nests."

The livers from the two dead embryos show a typical leukaemic picture (Fig. 13). The infiltrating round cells are primitive, and can only be designated by the term haemocytoblasts. An examination of the heart blood from these two embryos shows in the case of the liver only implant a large number of primitive cells which appear to be differentiating into erythroblasts, i.e. erythroleucosis, and in the second case (liver + spleen implant) a typical myeloid leukaemia (Fig. 14). Blood and tissues from the living (spleen only implant) embryo show no deviation from the normal.

Experiment 29.—Three 5-day embryonated eggs received 0·05 ml. blood from the living embryo which had the leukaemic spleen implanted on the chorioallantois. The blood was simply dropped on to the exposed membranes. One embryo was examined on the 17th day and found to be normal, as also was the membrane. The other two hatched out normally on the 21st day, and remained normal until they were killed 18 months later.

Experiment 30.—Four 3-day embryonated eggs were implanted on the chorio-allantois with fragments of tumour tissue from a case of multiple lymphocytoma of the liver of a R.I.R. hen. The bird was destroyed and immediately opened, and the viscera were exposed to the air in the post-mortem room for about 2 hours before the case was seen, but the liver surface was seared with a hot spatula at the time of implantation, and fragments taken from within the organ with sterile knife and forceps. The actual implants were made about 2½ hours after the fowl was destroyed. Broths inoculated with tumour material at the same time were later seen to be sterile.

Nine days later two embryos were found to be dead. They were congested and a blood examination showed large numbers of leucocytes, which were degenerated as the result of autolysis, which proceeds rapidly at incubator temperature. The third chick hatched normally and survived for two days. Blood films and sections of liver and spleen from this case were normal.

Experiment 31.—Six 10-day embryonated eggs were implanted with fragments of a teratoma arising in the right kidney of a Light Sussex hen. The tumour weighed 580 g. (Fig. 15).

Four days later three of the embryos were found to be dead and congested. They were bacteriologically sterile, as was the original tumour. Of the remaining eggs, two contained tumours 5 mm. diameter, and well supplied with blood vessels from chorioallantois. The growths were surrounded by an intense haemorrhagic zone (Fig. 16). The other egg contained an apparently normal embryo, and the membrane was free from tumour implant.

Histologically the original tumour was composed of embryonic renal tubules reminiscent of the developing metanephros, a connective-tissue moiety with a sarcomatous appearance, plain muscle fibres, circular islands of cartilage, and tubules composed of ciliated mucous secretory cells suggesting tracheal or bronchial epithelium (Fig. 17). There were large areas of haemorrhage and necrosis.

The two cultivated tumours (4 and 7 days respectively) both show a vascular sarcomatous-looking connective-tissue stroma, in which are embedded a few scattered tubules of epithelial cells, resembling those of embryonic kidney. Numerous bundles of plain muscle fibres are scattered throughout the tumour. There is a distinct ectodermal proliferation of the membrane adjacent to the tumour, and many " cell nests " are present in this region, as well as in the mesenchyme investing the growth (Fig. 18).

The results of the preceding experiments are summarized in Table I. It will be seen that the reaction of the chorioallantois has been somewhat arbitrarily divided into simple ectodermal proliferation and " cell nest " formation. These reactions of the membrane ectoderm differ only in degree; since simple proliferation may or may not be followed by " cell nest " formation, according to the nature of the stimulus.

DISCUSSION.

It is not necessary to emphasize the importance of an easy method of cultivation of tumours. Until the egg cultivation techniques were applied to cancer research tumours could only be propagated and studied in the laboratory either by direct homologous implantations or tissue culture. The first method is not

TABLE I.

Implant.	Virus.	Exp. No.	Reaction of adjacent membrane: Ectodermal proliferation.	Reaction of adjacent membrane: "Cell nest" formation.
Rous I sarcoma	+	1	+	+
		2	+	+
		3 (infected)	−	−
		4 (virus only)	+	+
Duran-Reynals sarcoma	+	5	+	+
—	Fowl-pox	6	+	+
—	Contagious papilloma (bovine)	7	+	−
Chemically induced mouse mammary carcinoma	— ?	10	+	+
		11	+	+
GRCH/15	— ?	14	+	+
Osteogenic sarcoma (dog)	?	18 (failed to grow)	−	−
Spontaneous adeno-carcinoma (chicken)	?	22 (infected)	−	−
		23 (failed to grow)	−	−
		24 (infected)	+	−
		25 "	−	−
		26	+	+
		27	+	+
Lymphoid leucosis (chicken)	+	28	+	+
		30 (embryos died)	Membranes unsuitable for examination.	
Embryonal nephroma (chicken)	?	31	+	+
		CONTROLS.		
Normal adult chicken :—				
Liver	—	— (failed to grow)	−	−
Spleen	—	— (infected)	+ (also endoderm)	+
Liver	—	—	−	−
Kidney	—	—	−	−
Ovary	—	—	−	−
Testis	—	—	−	−
Spleen	—	—	−	−
Chick embryo :—				
Gut	—	—	+	+
Liver	—	—	+	+

dependable, since in many cases it has been observed that tumours will only grow successfully in hosts closely related to the donor, a condition not always easy to fulfil, especially with unusual tumours growing in unusual hosts. The second method of tissue culture is involved and requires an elaborate technique, and has the great drawback that frequently only one component of the original neoplasm grows, while the rest, e.g. the stroma, tend to disappear.

When the yolk-sac technique was described in 1942 by Taylor *et al.* it seemed from their paper that a very easy and reliable method of cultivation of heterologous neoplastic tissue was at last available to workers in the cancer field. Other workers (Hungate *et al.*, 1944 ; Heilman and Bittner, 1944 ; Bittner *et al.*, 1945) also tried this method, and claimed good results with the particular tumours with which they worked—mainly derived from the rat and mouse.

The method, however, was not found so reliable by Twombly and Meisel (1946), who reported a very high mortality in their embryos, a disappointingly small percentage of successful takes, and complete failures with several types of tumour.

These findings have been confirmed by the present investigation, where, in a number of experiments, in which 157 eggs were injected with various mouse, rat and chicken tumours, both spontaneous and chemically induced, growths developed in the yolk-sac in only five instances.

It should not be thought, however, that the method is therefore of little value. In some hands it appears reasonably reliable, whilst in others not so reliable, and certainly wasteful of fertile eggs.

Certain results obtained with this yolk-sac inoculation technique are interesting and are being further investigated. For instance, in a number of cases embryos developed haemorrhages and died at varying periods subsequent to injection of either tumour suspension or tumour filtrate. These deaths were not always due to bacterial contamination, and could tentatively be ascribed to some lethal agent such as a virus, though the possibility that death was caused by the introduction of foreign proteins into the food supply of the embryo must not be overlooked. More experiments involving sub-inoculation with tissue filtrates and extra-embryonic fluids from dead and haemorrhagic embryos should help to settle this point.

The chorioallantoic method has been found to be most successful in the case of the cultivation of avian tumours associated with viruses. Thus, the Rous I sarcoma and the Duran-Reynals sarcoma give the biggest growths in the membrane.

These are both rapidly growing tumours, and therefore would naturally tend to give better results in the short period (never more than 14 days) of cultivation. Slow growing tumours usually give disappointingly small growths, whilst normal adult tissues may fail to grow and be absorbed.

In the case of the Rous and Duran-Reynals sarcomata, the virus stimulates the mesenchymal tissue of the chorioallantois to participate in the general malignancy, thus augmenting the size of the implant. Of more interest, however, is the observation, originally by Keogh (1938), that the Rous virus also appears to stimulate the ectodermal cells of the chorioallantois to proliferate in an apparently uncontrolled manner. He speculates on the possibility that the virus may be carcinogenic, not only for mesenchymal tissue, but also for epithelium under certain conditions. Ectodermal proliferation due to Rous virus has been confirmed in the present investigation, and the pathological changes in the membrane ectoderms produced by the Rous and Duran-Reynals tumours have been compared with those produced by dermatrophic viruses such as those of fowl-pox and contagious papillomata of cattle, also with similar changes noted in the membrane adjacent to certain implanted spontaneous tumours of the chicken. As a check on these observations a number of experiments were performed with chemically induced tumours of the mouse, rat and chicken; also with normal adult and embryonic chicken tissues.

The significance of the "cell nests" or "epithelial pearls" is interesting, resembling as they do transverse sections of infiltration cores of epithelial tissue which characterize squamous-cell carcinoma. Similar lesions were noted by Huxley and Murray (1924) adjacent to the fragments of chick embryos implanted on the chorioallantois.

It is well known that embryonic tissue extracts contain growth-promoting substances, and for this reason are frequently added to tissue culture media. Claude (1938) showed that a fraction could be isolated from chick embryos which

possessed the same physical and chemical properties as the purified active fraction of the Rous I sarcoma. Despite these similarities, however, the embryo extract failed to produce tumours in susceptible chickens.

In this connection it is interesting to note that Earle (1943) noticed an apparent malignant transformation of mouse fibroblasts grown on a fibrin clot and bathed in horse serum and chick embryo juice.

These observations, together with the production of ectodermal proliferation and cell nest formation found to be associated with so many chorioallantoic implants of tumours in the present investigation, gave rise to the conception that tumours and embryonic tissue might contain growth-promoting substances capable of causing the overgrowth of the ectodermal layer of cells in the embryonic membrane.

If this proved to be the case it might be possible to demonstrate some relationship between the intensity of the membrane reaction and the type of tumour implanted, especially from the aspect of virus content. No mention of " epithelial pearls " is made by Keogh (1938), or by the numerous other workers who have studied ectodermal lesions of the chorioallantoic membrane induced by viruses.

A number of experiments were devised in order to test this hypothesis, and especially to study the membrane reaction to a spontaneous liver carcinoma of the duck, which is described in a separate paper.

Table I shows that the ectodermal lesions do not appear to be as specific as was hoped. In all cases where tumours known to contain virus, or filtrates of such tumours, or dermatrophic viruses, were placed on the membrane, proliferation of the ectoderm took place. In some instances eosinophilic " inclusions " were observed, but the nature of these is not clear, except of course in the case of fowl pox. In the main, virus alone caused simple proliferation without the formation of " cell nests."

In general those tumours which failed to grow subsequent to implantation and were absorbed did not cause any detectable lesions in the membrane ; similar results were obtained for normal adult tissues. Also in several cases, although not invariably, an accidental infection of the membrane with bacteria did not result in ectodermal proliferation.

The spontaneous chicken tumours which grew successfully all caused proliferation and the formation of " cell nests."

It was thought possible that chemically induced tumours, being presumably free of virus, might give a different membrane reaction. The G.R.C.H./15 (1:2:5:6-dibenzanthracene) induced fowl sarcoma did not give rise to lesions in the membrane adjacent to the growth, but there were proliferative changes in the ectoderm investing the tumour, and atypical " cell nests " were seen in the interior of the growth. The two chemically-induced mammary tumours of mice, however, both gave rise to typical reactions in the adjacent membranes.

The induction of leukaemic myeloid leucosis in one embryo, and erythroleucosis in a second embryo following implantation of liver and spleen from a case of leukaemic lymphoid leucosis, and of an undiagnosed leukaemia in an embryo following the implantation of lymphocytoma of the liver are of interest, and appear to confirm the findings of Pierce (1942). Uhl, Engelbreth-Holm and Rothe Meyer (1936) report that the virus from a case of stem-cell leukaemia in the chicken may induce myeloid leucosis, erythroleucosis or even sarcomata

according to the developmental stage of the cell (haemopoietic or a near relative, either ancestral or descendant) which is attacked.

SUMMARY AND CONCLUSIONS.

Various *in vivo* methods of cultivation of spontaneous and chemically induced avian and mammalian tumours have been tried, and the best results have been obtained with the chick embryo chorioallantoic membrane method.

Virus-associated tumours grow best, followed by the chemically induced tumours. The majority of spontaneous tumours give disappointingly small growths.

It has been shown that the capacity for inducing ectodermal proliferation in the chorioallantois is not confined to tumour viruses, or to fowl pox and other non-tumour associated viruses, but occurs in the membranes adjacent to the majority of implanted tumours, whether spontaneous, virus associated, or chemically induced; or even occasionally in response to bacterial growth. Attention has been drawn to the tendency of the proliferating ectoderm to form "cell nests" or "epithelial pearls," resembling those occurring in squamous-cell carcinoma, and the significance of this has been discussed.

The induction has been described of two distinct leukaemic conditions in chick embryos as the result of implanting tissues from a third type of spontaneous leukaemia in a fowl, and has been briefly discussed.

I wish to express my gratitude to Professor A. Murray Drennan, of the Pathology Department, University of Edinburgh, for his interest in this work, and for the numerous suggestions as to the manner of development of the investigation.

All the fertile eggs were obtained from Dr. A. W. Greenwood's flock of Brown Leghorns, and I have pleasure in recording my appreciation of his co-operation in this respect, and for much help in other directions.

The virus associated and chemically induced tumours were supplied by Dr. J. G. Carr, formerly of the Institute of Animal Genetics, University of Edinburgh, to whom I am much indebted.

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INTRANASAL ADMINISTRATION OF MAMMARY TUMOUR MILK FACTOR.

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ONE of the surprising elements in Bittner's discovery of the "Milk Factor" is the apparent entry of the virus into the bodies of the young mice via the alimentary canal. It is, however, obvious that during suckling a small amount of milk may also enter the nostrils, and that the nasal mucosa may offer an alternative port of entry. It also seems possible that the milk factor might be destroyed by digestion in the stomach. If this were so it would be difficult to recover milk factor from the stomach contents of suckling mice. An experiment designed to demonstrate the presence of milk factor in the stomach was carried out between 1941 and 1943 by my colleagues, Dr. W. E. Gye and Dr. R. J. Ludford, who have permitted me to refer to their (unpublished) work. They removed the stomach contents from suckling mice of high cancer lines, extracted with saline and centrifuged them to throw down any cellular material. The supernatant fluid was then injected into suitable hybrid mice, free of the milk factor and known to develop mammary cancer when treated with it. The result was entirely negative in the 40 mice so treated Unfortunately the experiment was not adequately controlled, in that none of the mice were tested for susceptibility by means of extracts known to contain the virus. The evidence, therefore, that the inoculum given to the 40 mice did not contain milk factor is strongly presumptive though not conclusive.

In the present series of experiments an attempt has been made to test the other possibility alluded to above, namely, that entry may be via the nasal

mucosa, and in this series of experiments, the details of which follow, it has been possible to obtain tumours in approximately 43 per cent of female hybrids (free of milk factor) after one to three intranasal instillations of a tumour extract; long continued dosage produced no tumours.

DETAILS OF EXPERIMENTS.

Mice.

Hybrids from mothers of a low cancer strain (C 57 Black) and fathers of a high cancer strain (either Strong A or R 3) were used.

Oestrin.

The hormonal stimulus was supplied by forced breeding of females, or by painting the skins of males with 0·01 per cent oestrin in chloroform twice weekly for 27 weeks.

Milk factor.

This was extracted from spontaneous tumours arising in the paternal strains by grinding 5 g. of tissue with sand, diluting with 20 c.c. of saline and spinning at 12,000 for 15 minutes. The supernatant fluid was used for the inoculations.

Experiment 1.

Sixteen 3-weeks-old hybrid mice (C 57 females × Strong A males—8 males and 8 females) were segregated as controls. These received no further treatment, but were observed until death or for 20 months. Fourteen similar newborn hybrids (5 females and 9 males) were each given 0·05 c.c. of tumour extract (Strong A) intranasally under light anaesthesia. All the females (both control and experimental) were forcibly bred as they grew.

Result.—No tumours in the controls. Two mammary cancers in the five inoculated females, confirmed histologically. One female died in 52 days. Two females negative after 20 months.

Experiment 2.

Eight litters (19 females and 14 males) of hybrids (C 57 females × R 3 males) segregated as controls and observed for 24 months. The second litters of the same parents (18 females and 8 males) were each given 3 intranasal inoculations of a tumour extract (R 3) at weekly intervals (first inoculation 0·05 c.c., later ones 0·1 c.c.), beginning at 14 days. Both the treated and the untreated females were forcibly bred, and the males were painted with oestrin as described above for 27 weeks.

Result.—No tumours in the controls. Nine mammary cancers in the 26 inoculated mice (8 in females, 1 in a male) confirmed histologically.

Experiment 3.

Sixteen female hybrids (C 57 females × R 3 males) were segregated as controls and observed for 20 months. Eighteen similar hybrid females (second litters of the same parents) were inoculated intranasally once a week for 12 weeks with

an extract of an R 3 sporadic mammary cancer (first inoculation 0·05 c.c., subsequent inoculations 0·1 c.c.). All the females were forcibly bred.

Result.—No tumours in the controls. No tumours in the inoculated females.

CONCLUSIONS.

1. Intranasal inoculations of milk factor derived from sporadic mammary cancers can induce mammary tumours in hybrid mice free of the milk factor.
2. Both forcibly bred females and to a less extent oestrin-painted males are affected.
3. The optimum dose is one to three inoculations of from 0·05–0·1 c.c. of tumour extract in the first 5 weeks of life.
4. Continued intranasal inoculations (up to 12 weeks) appear to show an inhibitory effect, but further work would be necessary to prove this.

AN ATTEMPT TO DEMONSTRATE NEUTRALIZING ANTIBODIES TO THE MAMMARY TUMOUR "MILK AGENT" IN MICE.

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IT is known from the work of Andervont and Bryan (1944) and Green, Moosey and Bittner (1946) that the milk agent is antigenic for the rabbit and the rat. If, as is probable, the agent, is a virus one would expect it to be antigenic for the susceptible species. To see if this was so a search was made for neutralizing antibodies in mouse sera. Our results were essentially negative, and almost half the animals perished in the fire at the Jackson Laboratory. One can therefore draw theoretical conclusions only with extreme caution. However, we are able to make certain technical suggestions that may be of help to other workers in the field.

MATERIALS AND METHODS.

Serum was obtained by cardiac puncture following death from carbon monoxide poisoning.

The "milk agent" was obtained from spontaneous C3H mammary tumours, saline being added to make a final concentration of 50 ml. per g. of tumour. Following clearing in the centrifuge at 3000 r.p.m. filtration was accomplished through a tested Berkefeld V filter.

In each experiment mixtures were made of serum plus saline, serum plus tumour filtrate, and saline plus tumour filtrate. These mixtures were incubated at 37° C. for 30 minutes. Thereafter 0·1 ml. was injected intraperitoneally.

The mice used as test animals were females of the cross (A ♂ × C57 black ♀) F_1 × A ♀, known as ABC. Forced breeding was carried out, and only females which bred are included in the data.

In Experiment No. 1, normal C57 black serum was used. In Experiment 2 normal A serum was put in a wax oven at 56° C. for 30 minutes and then cooled prior to incubation. In Experiment 3 normal A serum was used. In Experiments 4 and 5 serum from hyper-immunized blacks was used. C57 black mice were injected at weekly intervals as follows: 0·1 ml., 0·2 ml., 0·4 ml., 0·8 ml., 0·8 ml., 0·8 ml. Owing to shortage of test animals the experiments were divided into two parts. In one the mice were bled 8 days after the last injection, in the other at 12 days. In Experiments 4*a* and *b* the serum was unheated, in Experiments 5*a* and *b* it was heated as in experiment 2.

RESULTS.

The results are shown in Table I. It will be noticed that in Experiments 1 to 3 the tumour filtrates were of approximately equal strength, whereas it seems to have been weaker in Experiments 4 and 5. The mammary tumours were all typical adenocarcinomas. As will be seen from the Table there were a number of leukoses, especially in the group receiving unheated A strain serum (Experiment 3). A few papillomata also occurred.

There is a suggestion of neutralization in Experiment 2. However, the probability of obtaining such a result by chance alone is of the order of 10 per cent. It is specially regrettable that this experiment was terminated, as a higher degree of significance might well have been obtained.

On the other hand, there is no suggestion of any neutralization in Experiments 4 and 5, where hyper-immune serum was used together with a relatively weak tumour filtrate. Had there been even weak neutralization one would have expected complete absence of tumours in the pertinent groups.

DISCUSSION.

The part played by circulating neutralizing antibodies is by no means clear in all virus diseases. Confining our attention to mice, it may be noted that Traub (1936) showed that immunity to lymphocytic chorio-meningitis was not accompanied by demonstrable protective antibodies. Of special pertinence to experiments with serum from hyper-immunized blacks may be the observations of Webster (1938) and Hodes and Webster (1938) with the virus of St. Louis encephalitis. Here a high degree of immunity developed about 6 weeks after vaccination, and had waned before antibodies appeared some 20 weeks after injection. It may be that we bled our mice too soon.

Possibly our technique was entirely inadequate to show any neutralization. However, identical techniques were used by Andervont and Bryan (1944) and Green, Moosey and Bittner (1946). Two sources of error suggest themselves: first that the tumour filtrate was too strong, secondly that our period of incubation was too short. Whilst union of antigen and antibody is usually very rapid, it is well known that virus-serum mixtures may dissociate on dilution unless incubated for some time (Andrewes, 1930; Salaman, 1938), the dilution here occurring after injection. Unfortunately the destruction of Experiment 2 (with

TABLE I.

Experimental procedure.	No. mice.	Eff. total.	No. tumours.	Perc. tumours.	Tumours by month. 6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	Other tumours.
(1) C57 black normal serum :																	
Saline + serum	30	27 (19)*	..	..	..	..	..	..	..	..	..	..	..	..	..	..	Papilloma, 4 months.
Serum + milk agent	30	30 (11)	8	26·6	..	..	..	..	1	2	4	1	1	..	..	..	Leukaemia, 13 months.
Milk agent + saline	30	27 (10)	5	18·5	..	..	..	..	..	1	1	..	2	..	1	..	..
(2) Heated A serum :																	
Saline + serum	30	28 (13)	1	3·5	..	..	..	..	..	..	..	..	..	1	..	..	Leukaemia, 11–14 months. (2 cases)
Serum + milk agent	30	24 (17)	1	4·2	..	..	..	..	..	..	..	..	1	..	..	..	Papilloma, 12 months.
Milk agent + saline	30	28 (14)	5	17·9	..	..	..	1	1	..	..	1	1	1	..	..	Papilloma, 15 months.
(3) A serum :																	
Saline + serum	31	27 (19)	3	11·1	..	..	..	..	..	..	1	1	1	..	..	..	Leukaemia, 12 months.
Serum + milk agent	32	30 (12)	7	23·3	..	..	1	1	2	1	1	..	..	..	1	..	Leukaemia, 10, 12 months (2 cases)
Milk agent + saline	31	26 (15)	5	19·2	..	1	1	..	..	1	..	1	1	..	..	..	Leukaemia, 13 months.
(4) C57 black immune serum :																	
Saline + serum	36	34 (28)	1	2·9	..	..	..	..	..	..	..	1	..	..	..	..	..
Serum + milk agent	36	34 (23)	2	5·9	..	..	..	..	..	..	..	..	1	1	..	..	..
Milk agents + saline	34	32 (16)†	2	6·2	..	..	..	..	..	..	..	..	..	2	..	..	..
(5) C57 black heated immune serum :																	
Saline + serum	34	31 (18)	1	3·2	..	..	..	..	..	..	..	1	..	..	..	..	Papilloma, 12 months.
Serum + milk agent	34	30 (13)	5	16·7	..	..	..	..	..	1	..	3	1	..	..	..	..
Milk agent + saline	34	31 (24)	1	3·2	..	..	..	..	..	1	..	..	..	..	..	..	Histiocytoma, 15 months.

* Number of mice tumour-free when destroyed by fire.
† 12 mice died of chronic pneumonia within tumour period.

heated A serum) must leave these fundamental questions unanswered. A strain mice are being constantly immunized since birth. It may be that this very prolonged stimulation is needed for the production of protective antibodies. The fact that the agent can be demonstrated in serum may be due to dilution following injection. If this is so, dilution of the serum prior to inoculation should give a greatly enhanced incidence of tumours.

SUMMARY.

1. An attempt was made to detect protective antibodies against the " milk agent " in mice. The experiments were largely terminated by the fire at Bar Harbor.

2. A suggestion of neutralization was obtained with heated high cancer (A strain) serum.

3. No suggestion of neutralization was obtained with serum from unheated A strain serum, unheated C57 black serum, or from heated or unheated serum from hyper-immunized blacks.

4. The significance of these findings is discussed in the text.

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INHIBITION OF THE CARCINOGENIC PROPERTIES OF URETHANE BY PENTOSE NUCLEOTIDES.

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URETHANE, which induces lung tumours in mice and rats (Nettleship and Henshaw, 1943 ; Jaffe, 1947 ; Cowen, 1947), also causes a notable decrease of malignant and normal leucocytes (Paterson, Haddow, ApThomas and Watkinson, 1946 ; Cowen, 1947). The present study was undertaken to investigate any correlation between the carcinogenic and leucopaenic properties of urethane.

The mixture of pentose nucleotides as prepared from yeast nucleic acids is used clinically in the treatment of agranulocytosis, and it has been found to elicit leucocytosis in mice (Parsons, 1945). Since pentose nucleotides have the opposite effect of urethane with respect to their action on leucocytes, the possibility that the carcinogenic effect of urethane might be antagonized by pentose nucleotides was considered.

MATERIALS AND METHODS.

The mixture of pentose nucleotides used was the commercial preparation, as supplied by Menley & James Ltd. in 10 c.c. sterile vials, each containing 0·7 g. of a mixture of the sodium salts of the four nucleotides of yeast ribonucleic acid preserved with 0·3 per cent cresol.

The mice used were the F_1 cross of the A and C 57 lines. These were chosen because A mice have a relatively high spontaneous lung tumour incidence (Bittner, 1939 ; Heston, 1940), and it has been observed that such strains respond more readily to the carcinogenic effect of urethane (Cowen, 1947). The hybrids would also tend to have a high spontaneous incidence, since the inheritance of spontaneous lung tumours in this cross seems to be due to a single dominant gene (Bittner and Little, 1937 ; Bittner, 1938).

Seventeen A × C 57 males approximately two months old were injected with the pentose nucleotides solution subcutaneously at the flanks, daily at mid-morning. The dosage was 0·1 c.c. for 3 days, 0·2 c.c. for 4 days, 0·4 c.c. for 1 day, and then a 0·1 per cent solution of urethane was substituted for the drinking water. After continuing to inject 0·4 c.c. of pentose nucleotides for a week the mice started to show ill effects. Both treatments were discontinued (after 2 weeks of pentose nucleotides and 1 week of urethane treatment) for two days, to rest the mice. On resuming treatments the dosage of pentose nucleotides was decreased to 0·3 c.c. daily. This rate of dosage was tolerated much better. Eight weeks from the time of the first injection the urethane solution was replaced by tap water, and four days later the injections of the pentose nucleotides were stopped.

As controls, 9 A × C 57 hybrid males, also 2 months old, were treated similarly with urethane only (Control 1). In order to ensure that the urethane was being administered well above threshold level, a further group of 11 identical control mice (Control 2) were similarly treated but for a period of 3 months, i.e. 5 weeks longer than Control 1 and the experimental mice.

All the animals were kept for 7 months from the beginning of treatment, and then sacrificed.

RESULTS.

Thirteen of the 17 mice treated with both pentose nucleotides and urethane, 8 of the 9 Control 1 mice and 9 of the 11 Control 2 mice survived the experiment. After separating the lobes, the total number of lung tumours in each mouse

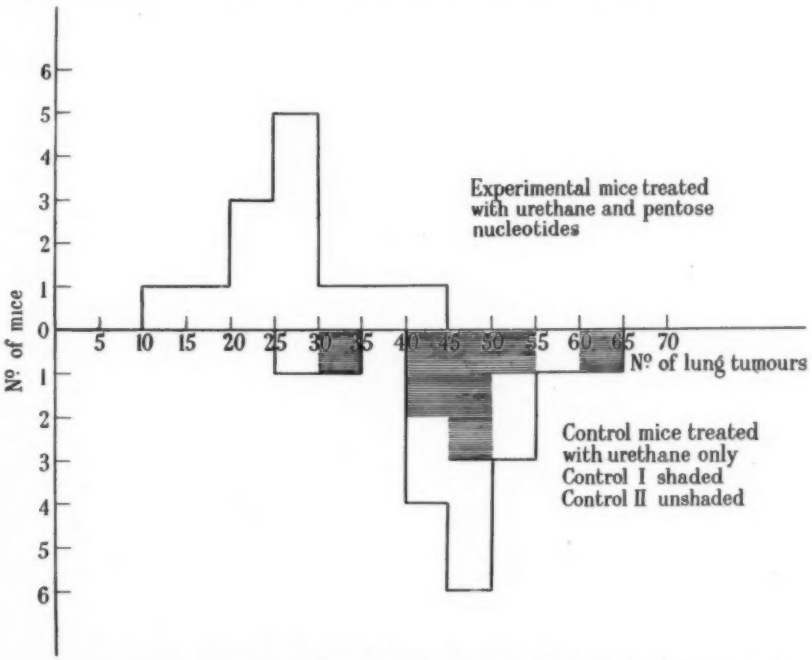

FIG. 1.—Frequency polygon showing number of lung tumours in A×C 57 mice treated with urethane and pentose nucleotides compared with A×C 57s treated with urethane only.

was counted with the naked eye and was plotted as a frequency polygon (Fig. 1). The shaded areas represent Control 1 mice. The figure clearly shows that a marked inhibition of the carcinogenicity of urethane was produced by the pentose nucleotides.

The average number of lung tumours per mouse caused by urethane in Controls 1 and 2 separately was 46 in each case, which was reduced to 26 in the

group receiving pentose nucleotides. This represents a decrease of 43 per cent in tumour incidence.

The highest number of lung tumours found in the pentose nucleotides treated mice, 42, was exceeded by 13 of the 17 controls which received urethane alone.

As already noted, the average number of lung tumours per mouse in Control 1 was the same as in Control 2. This indicates that the urethane was most probably exerting its maximum carcinogenic activity during treatment with the pentose nucleotides. It is to be suspected that with a lower dosage of urethane, the pentose nucleotides treatment might have been even more efficient in inhibiting lung tumours.

DISCUSSION.

The results of this experiment show that there seems to be some connection, direct or indirect, between the carcinogenicity of urethane and its leucopaenic properties, since the carcinogenic effect is inhibited by a substance which causes leucocytosis.

The exact mode of action of urethane, as a carcinogen specific for lung, remains obscure. Various possibilities exist ; one is that urethane, resembling so many normal tissue components, substitutes for one or more of them and upsets metabolism. Haddow and Sexton (1946), in their work on the inhibition of the Walker rat tumour by urethane, suggest an upset of purine metabolism. This would fit in with the present results, since pentose nucleotides, here shown to antagonize the action of urethane, contains purine and pyrimidine compounds.

If lung tumours were not directly the effect of a purine metabolism upset, then a second possible path of action presents itself. The deviation of purine metabolism may be responsible for the leukopaenia noted (Paterson *et al.*, 1946 ; Cowen, 1947), which in turn lowers the resistance of the animals to some latent organism in the lung which would be responsible for the tumours (Nettleship and Henshaw, 1943). This involves a direct connection between the carcinogenicity of urethane and its leucopaenic powers. It is interesting to note that narcotics and certain other substances chemically related to urethane are neither carcinogenic for lung nor have they been noted for leucopaenic properties (Larsen, Rhoads and Wood, 1946 ; Cowen, 1947). The pentose nucleotides might therefore act by directly supplementing the normal cell purines, rendering the urethane ineffective as far as metabolism deviation is concerned ; or it may independently of this purine mechanism produce leucocytosis, thus neutralizing the leucopaenic action of urethane. In both cases antagonism of the carcinogenicity of urethane by pentose nucleotides is explained whether it acted by a straight metabolic upset or through a virus or similar organism.

Further important information could be obtained if each of the four nucleotides, in the mixture used, were separately tested for anti-carcinogenic effects, noting if the same ones were responsible for leucocytosis, since this property varies between them (Parsons, Gulland and Barker, 1947). At the present time the pure nucleotides are not available in the quantities required for work of this nature. The " purine deviation " hypothesis above would suggest that the purine or pyrimidine bases alone might be anti-carcinogenic.

It has also been suggested that urethane acts as a carcinogen by merely speeding up a natural process (Cowen, 1947). This was concluded, since the

incidence of urethane induced lung tumours bears a relationship to the incidence of spontaneous lung tumours in various inbred lines of mice. In this event, pentose nucleotides would be expected to have an inhibitory action on the formation of spontaneous lung tumours.

SUMMARY.

1. Pentose nucleotides, when injected into mice receiving treatment with urethane, decreases almost by half the number of lung tumours caused by it.

2. Possible modes of action of urethane as a carcinogen for lung were discussed, especially with respect to its leucopaenic action.

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THE INDUCTION OF MUTATIONS BY A CARCINOGEN.

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THE idea of somatic mutation as an explanation for the origin of cancer has had a long and interesting history. Its original use as an interpretation of cancer is usually associated with the names of Murray and of Boveri. These men arrived at the somatic mutation conclusion from different fields, Murray from experimental cancer research, and Boveri from his classical observations in experimental embryology. A good description of the origin of cancer was formulated by Murray (1908). At that time he stated: "The existence of such tumours, the biological characters of which are retained through long periods of propagation, shows that the cellular transformation which initiates carcinomatous growth may take place in varying degrees. The impress which the cells receive, at this time, while permitting of great histological variations in their descendants,

colours permanently their whole biological behaviour. This biological alteration is of such a kind that the cells are able to take up nourishment, increase in size and multiply indefinitely. They acquire an individuality and powers of resistance to injurious agencies superior to those of normal tissue elements."

Boveri's idea on the nature of the origin of cancer stemmed from his observations on atypical mitoses. He had suggested the possibility that by this means an unequal distribution of the chromosomes would ensue and thus lead to uncontrolled growth. He was loath to expand upon this concept until Aichel (1911), using Boveri's own observations on the development of the sea urchin egg, arrived at the conclusion that a malignant tumour was caused by a fusion of a tissue cell with a leucocyte. Boveri's concept has not received wide recognition. The essential feature of the idea is expressed in his own words (Boveri, 1929): "The essence of my theory is not abnormal mitosis, but in general, a definite abnormal chromosome-complex. However this may arise, the result would always be a definite tumour. Beside the multipolar mitoses, which might depend either on a simultaneous multidivision of the centrosome or on distorting the parallelism between the division of the centrosome and the cell-division, asymmetrical mitosis should be chiefly considered for the origin of tumours. Indeed, according to analogy with certain occurrences in sea urchins, these would depend on a lack in certain chromosomes of the power to divide; this would result far more surely in tumour than would multipolar mitoses, which depend on chance for the distribution of their chromatin. Agencies which would act most directly would be those that have the power of destroying definite chromosomes of a cell, while leaving the others uninjured." Boveri, therefore, saw quite distinctly that irrespective of the mechanism of origin, the disturbed chromatin content is the essential feature in cellular physiology, including the abnormal condition of a tumorous growth. The presence of multipolar and other abnormal mitotic figures in tumours and cancers had been investigated previously by von Hansemann (1890, 1891*a*, *b*). Von Hansemann had used these observations in the origin of his concepts of *prosoplasia* and *anaplasia*. The pleomorphism of cancer cells and the irregular atypical mitoses so characteristic of their nuclei had been observed and carefully studied, however, since the middle of the nineteenth century.

Whitman (1919) wrote a critical review of von Hansemann's theory of anaplasia in the origin of cancer in terms of modern genetics. Tyzzer (1916) expressed the opinion that a somatic mutation may be involved in the origin of cancer. Tyzzer stated, "There are marked differences in the behaviour of various tumours on transplantation in given classes of mice. Even tumours arising in homogeneous races show such differences, and this may be attributed to the acquisition of new characteristics by the soma which are manifested in the development of the tumour. The tumour, since it breeds true with respect to these characteristics in the course of artificial propagation, may be regarded as a modification of the somatic tissue which may be termed somatic mutation."

At the present time it may be well to indicate two aspects of the genetic problem in relation to the origin of cancer. These are (1) susceptibility and resistance to the spontaneous, the transplanted and the induced tumour—an inherited constitutional state or states in which the germ plasm is definitely involved, and (2) the origin of the neoplastic lesion by a conversion, somehow or other, from a pre-existing normal somatic tissue—a somatic mutation. This

actual process of somatic mutation may either be conditioned or under the control of an inherited or germinal influence, or entirely independent of such intrinsic determination. This distinction between the intrinsically determined susceptibility and resistance to cancer and the somatic origin by mutation is not always kept clearly in mind. As a matter of fact some investigators express the opinion that the two genetic phenomena are entirely antagonistic to each other. For example, a very recent article by Bauer (1948) of Heidelberg discusses at length that susceptibility to cancer in the human population is not inherited, and at the same time elaborates on the development of the concept that cancer arises as a somatic mutation. Bauer states, " The decisive importance of inherited characteristics which give rise to cancer is entirely disproved by the experiment of nature with the hereditarily identical and the hereditarily different fraternal twins." Bauer has been interested in the somatic mutation hypothesis for many years, having published his first article in 1928 (Bauer, 1928). Bauer (1948) concludes, " Thus the mutation theory of tumour development is an interpretation of all cancer phenomena adequate for the clinical, morphological, biochemical, and in particular genetical facts." A similarly expressed conflict between the germ plasm and the somatic mutation idea is expressed by Dunning, Curtis and Bullock, in which they arrive at the opposite conclusion in their extensive studies of induced sarcomas in the livers of rats, in that they conclude that susceptibility to the sarcoma condition is inherited, but that an ill-defined somatic mutation may also be involved in the origin of the lesion. This antagonism is, however, not as sharp as would be indicated by the title of their paper, "The Respective Rôles of Heredity and Somatic Mutation in the Etiology of Tumors Induced by Parasites and Chemical Irritants ' (Dunning, Curtis and Bullock, 1937). In 1933 these investigators had concluded : " Possibly when more is known about the etiology of other tumours for which there appears to be an inherited susceptibility and when the expression of genetic factors in the cells and tissues is better understood, it will be found that in the case of all neoplasms in all species the initial cell change occurs by a process analogous to somatic mutation, and that heredity factors determine this change only in so far as they influence longevity and the susceptibility of an individual to some specific irritant or condition which is favourable to mutation " (Curtis, Dunning and Bullock, 1933, 1934).

Their idea of the nature of this somatic mutation process in the origin of cancer is similar to the one expressed originally by Strong in 1926, who concluded : " This mutational process may be either a change or shifting of a complete chromosome or chromosomes, or a change or changes within a chromosome or chromosomes (genic), or it may be even cytoplasmic in nature. By mutation I merely mean to use the term in its broadest sense, that is, a change or shift within the genetic or internal constitution that results in definitely clear-cut or discernible differences in behaviour or structure that are perpetuated by the process of heredity (in this case, cell division)." (Strong, 1926*a*, *b*). That a tendency to undergo somatic mutation may have a definite genetic basis in normal tissues has been discussed by East (1917). This phenomenon has been encountered especially in plants, but also has been reported in the higher animals. An early example of a genetic influence on somatic mutations is discussed by Whitman (1919). He states, "A somewhat different condition is presented by the ' bud variations ' occurring in plants, and consisting in the production of

unlike flowers on different branches of the same plant, e.g. a yellow chrysanthemum carrying white flowers on one branch. It is obvious that, since the colour of the flower is known to depend on definite factors, the development of a branch carrying flowers of a colour different from that of the flowers on the other branches could only arise through the failure of the factors to pass over, during mitosis, into the cell from which that branch developed. The branch, in other words, represents a somatic mutation due to asymmetrical mitosis."

Many tumours have been described in a variety of genetic material. Many of these tumours are pigmented and occur especially in the early development of the individual, and some are associated with or are identical to lethal mutations. These have been recorded by Stark and Bridges (1926), Wilson (1924) and others in drosophila, by Federly (1936) in lepidopterous larvae, and by many other investigators. Most of these tumours are inherited by a multiple factor complex and, in the opinion of T. H. Morgan, expressed to the author many years ago, at least one of the drosophila tumours arose as a mutation. Time alone prevents the further discussion of this interesting genetic material and its bearing on the somatic mutation hypothesis.

The next advance in the somatic mutation idea in relation to neoplastic lesions was undertaken by the present author, in 1918. From that time onward until 1930, a considerable amount of evidence was accumulated from the study of the transplantation of adenocarcinomata of the mammary gland in mice that eventually provided *the first experimental evidence* that a somatic mutation may be involved in the origin of cancer (Strong, 1926*a*, *b*). Some of these data have been published, but a considerable amount has not. The development of this experimental approach to the nature of the origin of cancer was as follows : Tyzzer and Little investigated the transplantation of a sarcoma that originally arose in a Japanese waltzing mouse. They found that the progressive growth of this transplantable tumour was dependent upon the simultaneous presence of multiple mendelizing units (from 12–14). Strong and Little, in 1920, and later in 1924, determined that two transplantable adenocarcinomata of the mammary gland in mice were dependent upon different genetic complexes for their continued growth. The dBrB tumour was dependent upon the simultaneous presence of two genes, whereas the dBrA tumour was dependent upon the simultaneous presence of these same two genes, but required the presence, in the host, of a third gene. The presence of the third gene had no detectable influence on the growth of the dBrB tumour. This genetic similarity and difference was obtained in the same series of mice in spite of the fact that the two tumours were histologically indistinguishable (Strong and Little, 1920 ; Little and Strong, 1924). The opinion of identical diagnosis was given by the late Dr. James Ewing, as well as by Prof. Francis Carter Wood. Upon this evidence, and more of a similar nature that was soon obtained, the genetic theory for the transplantation of tumorous tissue was formulated in 1924. The original statement was that " the fate of the implanted tumour tissue when placed in a given individual (host) is brought about by a reaction between the host, determined to a large extent by the genetic constitution, and the transplanted tumour cell, controlled to some extent by its genetic constitution." The next experiment that advanced this genetic concept of the nature of cancer was obtained by the author and published in two papers (Strong, 1926*a*, *b*). This observation was the obtainment of sudden sharp changes in the transplantability, an increased growth rate, and, eventually,

invasive and metastatic capacities of the same transplantable tumour. The phenomenon of sudden change occurred many times while the problem was being investigated, but only one illustration will be given here. A third spontaneous tumour that arose in a dilute brown mouse (given symbol dBrC) provided evidence that six genes were involved originally in the growth of this tumour when transplanted into a series of suitable F_2 individuals derived from a cross between mice of the dilute brown stock and mice of a totally unrelated stock, the well-known A stock. Growth rates on the various transplantable tumours derived from the dBrC tumour were determined. After several transplant generations a tumour was obtained that grew so rapidly that it was considered out of the normal range of growth for the original transplantable tumour. When the derived transplant was tested it was found that now its growth was dependent upon the simultaneous presence of only two genes. This sudden acquirement of growth capacity also resulted consequently in a new transplantability characteristic. Thus the action of four genes in the genetic constitution of the host were no longer necessary for the successful transplantation of the tumour. A second sudden change subsequently took place, and a new derived (mutant) type of tumour would now grow in 75 per cent of all inoculated F_2 mice (observation $75{\cdot}00 + : 23{\cdot}00 - \pm 2{\cdot}79$). The probable error difference between this new ratio and the actual observation for the original dBrC tumour was $58{\cdot}13$ per cent $\pm$ $3{\cdot}67$ or $15{\cdot}84 \times$ P.E. This new derived transplantable tumour continued to give a 3:1 ratio in a series of F_2 mice for several transplant generations. Eventually a new sudden change took place at which time the new mutant type grew in practically all F_2 mice (observation $243{\cdot}00 + : 2{\cdot}00 - \pm 0{\cdot}94$). These new data in the F_2 deviated from the ratio obtained with the original dBrC tumour as follows: $80{\cdot}78$ per cent $\pm$ $2{\cdot}48$ or $32{\cdot}57 \times$ P.E. There can be no doubt that sudden genetic changes are taking place somewhere in the relationship between the host and the transplanted tumour. Since the mutant types and the original transplantable tumour were being compared in the same series of F_2 mice, these sudden changes could not be taking place in the host but must be taking place within the tumour cell (Strong, 1926*a*, 1926*b*). Another point that should also be emphasized is the fact that when the derived mutant transplantable tumour would grow in all F_2 mice derived from a cross between the dilute brown and the A stocks, then, and not till then, would it grow in all mice irrespective of genetic relationship. In other words, it had lost all characteristics of tissue specificity. The transplanted tumour would grow invasively at a tremendous rate and would metastasize extensively. Thus evidence was obtained that a highly specific, relatively benign, slow-growing transplantable tumour had progressively and suddenly acquired periodically new biological characteristics until finally it had been converted over into a carcinoma. The early sudden changes in the transplantability of the tumour were not accompanied by any changes in histological appearance. Toward the end of the series, however, especially at the terminal non-specific state, the tumour had become more cellular and had lost most of its original adenocarcinomatous arrangement.

Another series of investigations was performed on the transplantation of the 13 spontaneous tumours that mouse F_1 79 gave rise to. This mouse was not only the common ancestor of the well known C, C3H, CBA, C121 and CH1 strains of inbred mice, but also gave rise to a wealth of neoplastic tissue as follows: All 13 of her spontaneous tumours of mammary origin were tested out by trans-

plantation in a series of suitable related mice. The data obtained from the study of two of these tumours, F_1Da and F_1Dg alone, have been published (Strong, 1929). Thus, in a series of mice that had been injected with both tumours, one in the left axilla, the other in the right axilla, the data were obtained as shown in Table I.

TABLE I.—*Tumour Reaction.*

	+ G + A.	re. G + A.	re. G re. A.	− G + A.	− G − A.
Number of individuals	235	76	77	115	347

+ indicates the progressive growth of the transplantable tumour until the death of the mouse, re. indicates temporary growth followed by complete regression, and − indicates complete resistance to the transplant.

Thus it is evident the two transplantable tumours were giving a different reaction when transplanted into the same series of mice. There were similarities of reaction, however, as well as differences. Some mice would grow both tumours progressively, whereas other mice would refuse to grow either, and some showed temporary growth of both tumours. But in the same experiment it was found that a large series of 76 mice would show only a temporary growth of the G tumour but a progressive growth of the A tumour, and another group of 115 mice would refuse to grow the G tumour but would grow the A tumour. Further data obtained from the transplantation of these two tumours (F_1Da and F_1Dg) are given in Table II.

TABLE II.—*Tumour Reaction.*

	+ G.	+ A.	− G.	+ A.	− G.	− A.	Total.	
Sex	♀ ÷	♂	♀ ÷	♂	♀ ÷	♂	♀ ÷	♂
Original negative strain	0	0	0	0	46	40	46	40
Orig. F_1 neg. ♀ × + G + A ♂	52	0	0	48	0	0	52	48
F_2 from above	85	82	78	82	228	222	391	386
F_1 ÷ G ÷ A orig. neg. ♂	36	30	0	0	0	0	36	30
F_2 from above	102	48	0	51	140	134	242	233

The study of these two tumours derived from the same mouse produced data that permitted the following conclusions. The two tumours, even in spite of the fact that they were derived from the same individual and were histologically indistinguishable, were indeed quite different. They were physiologically different, and presumably genetically different also. If one assumes, for the sake of argument, that one of these tumours possesses the same genetic constitution as the mouse tissue from which it arose, then the other tumour tissue cannot have it. This assumption is a valid one, since the recent interpretations of histogenesis would lead us to the conclusion that qualitative cell-divisions probably do not occur in animate forms. Every cell of the adult body is supposed to be endowed with the same genetic potentialities. Since, therefore, one of the tumours must have a different genetic constitution from the mouse tissues from which it has originated, it must have deviated, presumably by some such a

process as somatic mutation, from the genetic constitution of the somatic tissue from which it arose. And if one tumour tissue has deviated from the genetic constitution of the somatic tissue from which it arose, then possibly all tumours undergo the same process in their origin.

The above phenomena especially of sudden changes taking place during the transplantability of spontaneous adenocarcinomas of the mammary gland in mice has been amply verified, not only by the investigation of the 11 other tumours derived from female $F_1$79 referred to previously, but also by many other tumours derived from other mice in my laboratory. All the phenomena of transplantation consistent with the genetic theory of transplantation were also verified by the independent work of Bittner (1931) and by Cloudman (1932*a*, *b*). Eight years later Little (1934) wrote a review on the genetic work on the transplanted tumour in mice in which he included a discussion of the somatic mutation idea. In this paper Little points out that he had outlined the genetic basis for susceptibility and nonsusceptibility to transplanted tumours in 1914 (Little, 1914). He states again in 1934 that " The bit of tumour tissue to be transplanted has a genetic composition, which is determined by that of the animal in which it arises." If this idea was exclusively true, then it is obvious that a somatic mutation could not have been involved in its origin since any mutational process should alter or change the original genetic state. It is indeed true that in the early transfer generations, the transplanted tumour derived originally from a spontaneous source does retain the characteristic of tissue specificity determined by the genetic constitution of the mouse, in that it will grow only in individuals which are genetically related to the mouse that gave rise to the tumour. But it has now been definitely proven that this phenomenon of tissue specificity is periodically and progressively lost by sudden changes resulting in simpler and simpler mendelian ratios, until eventually the tumour retains no or very little tissue specificity which it originally had. These sudden or mutational changes have always produced more and more malignancy, as determined by percentage of takes, growth rate and eventual invasive and metastatic characteristics. It has also been indicated that no two tumours, even though they be derived from the same mouse and present the same histological appearance, ever showed the same mendelian ratio when transplanted into a series of appropriate F_2 individuals. When two transplantable tumours were derived from the same mouse, some of the genetic complex involved in their successful transplantation is common to both tumours, whereas some are unique to either the one or the other tumour. Similarity of genetic transplantability complex indicates genetic relationship of origin for tumours. In this particular Little's concept of 1914 is therefore partially correct. It is also evident that the more recently chemically-induced tumours show, from their very origin, on an average, more malignancy, and thus less tissue specificity than do the spontaneous tumours that arise in the same series of inbred or hybrid mice. It is probably true, although at present impossible of proof, that there is some genetic mechanism within cells that determines to some extent normal cell relationship, but that under certain conditions this controlling genetic mechanism alters or changes, and by so doing permits an uncontrolled or cancerous condition to arise somewhere in the biological system (the organism). This phenomenon of break of co-ordinating influence of a definitive part is, I believe, the essential feature in the origin of cancer. The fact that the tumour or cancer grows progressively is of secondary importance,

following the experimental evidence reported by Strong (1926*a*, *b*), and verified later by Bittner (1931) and by Cloudman (1932*a*, *b*).

Little (1941) stated the problem in different terms from those he used in 1914. He now states: "In accepting as a working hypothesis the statement that cancer fulfils the requirements of a somatic mutation, namely, a sudden change which is perpetuated in succeeding cell generations, it must be noted that there are already on record two distinct general types of somatic mutation, either of which might be involved in the appearance of cancer. One of these is the result of internal environmental change directed by one or more genes. The other has a sporadic etiology dependent on various influences which affect individual cells or groups of cells directly. In the first category belong such cases as mutations in colour genes in rodents described by Castle, Pincus, Bittner and others. One of Castle's cases is particularly interesting in that it shows several cases of mutations occurring in several generations of a single family of rabbits, thus indicating a definite genetic basis. Cases of the second type have been recorded as the result of exposure to radio-active agents." Lockhart-Mummery (1934) has published a book on mutations and cancer, and there have been several other articles dealing with this problem. These cannot be discussed at this time.

The discovery that carcinogenic compounds can induce mutations when injected into suitable experimental animals has not originated the idea that cancer may arise by a process of somatic mutation, but rather has reopened interest in an old concept. The essential features of the historical development of this mutation concept have been outlined above. The problem has been discussed by many investigators. Some experimental evidence, although of an indirect nature, has been obtained, and it is clear that there is no evidence contrary to such a somatic mutation concept. The first criticism of such a concept that cancer may arise by some such a mutational process was expressed to me more than 25 years ago by a very distinguished geneticist, who stated that "you cannot prove that cancer arose from somatic tissue through the process of somatic mutation because you cannot hybridize cancer tissue to normal tissue from which it arose." It is indeed gratifying that in the last issue of *Sigma XI* quarterly this same distinguished geneticist (Sturtevant, 1948) has now expressed the opinion that the indication of a gene can be determined by a method other than by the process of hybridization.

A different biological interpretation of the evidence bearing upon the origin of neoplastic growths has been expressed by Dahlberg (1940). He states that "if tumours are regarded as descendants of a cell which has undergone mutation, it is understandable that tumour cells should show features differing from what is normal. Possibly we may also understand the autonomous character of the tumour tissues. On the other hand, the somatic-mutation theory hardly explains the embryonic features of malignant tumours; and it does not explain why growths are more frequent in older than in younger individuals, because it has not been observed that mutations in cells of the gonads are more frequent in older than in younger persons. Nor does it help us to understand why external stimulation should give rise to tumours, though it is possible to suggest more or less plausible analogies. Thus X-ray treatment may give rise to formations of tumours in the cells of the body and to mutations in the germ cells. A detail of particular interest is the occurrence of abnormalities among the chromosomes both in cells which have undergone mutation and in tumorous cells. The muta-

tion theory explains certain features of the tumours, but leaves other and essential features unexplained. If we want to explain the genesis of malignant tumours with the help of analogies to known biological phenomena of more general occurrence, it seems to the writer that another possibility lies nearer to hand ; and it is the object of the ensuing discussion to indicate the possibility of exploring some conclusions suggested by following out its consequences. The development of tumours may be compared to vegetative reproduction."

Whether the concept of vegetative reproduction will explain more phenomena concerning the origin of cancer than does the somatic mutation idea should perhaps be more fully discussed. This cannot be done at the present time.

The induction of mutations by carcinogens has led to the conclusion that " all carcinogens are mutagens and all mutagens are carcinogens." This expression has been used by the present author, by Dr. Demerec, and very recently by Prof. Bauer of Heidelberg. It is the purpose of this paper to analyse the present status of the problem in order to see whether this conclusion should now be accepted or discarded.

Germinal mutations and other biological variants induced by carcinogenic compounds have been reported in mice by Strong and by Carr. Hollander, working in my own laboratory, has also obtained similar results following the intraperitoneal injection of methylcholanthrene into pregnant mice that had been outcrossed to males of other inbred strains. This procedure of Hollander has induced germinal mutations which appeared for the first time in mice of the ensuing untreated F_3 generation. Prof. Tatum of Yale has also obtained a few mutations in neurospora with a derivative of methylcholanthrene and concludes that, in this species, methylcholanthrene is a mild mutagen. Dr. Demerec has reported data obtained on mutagenesis with many carcinogens and closely related non-carcinogenic chemicals in drosophila. According to him these compounds have been studied in conjunction with aerosol and other detergents. By this means he has reported many mutations (notably sex-linked lethals) with the carcinogenic hydrocarbons, and has stated that he has obtained a few chromosome breaks in his experimental material. It is an established fact that the term mutagenic is not an absolute characteristic for any physical or chemical agent. For example, it may be said that X-rays are a powerful mutagen. They are very effective in inducing mutations in drosophila and in neurospora, and to a certain extent in maize, but do so very poorly in mice. On the other hand, ultra-violet light appears to be more effective in inducing mutations in maize than in drosophila. Again it must be taken into consideration that the different physical and chemical agents bring about mutations in various ways. X-rays break chromosomes, thus leading to rearrangements of parts, transpositions and inversions, to deletions, etc. Ultra-violet light, however, seems more effective in inducing point mutations in both corn and drosophila, possibly by providing a specific wave-length, 2600°, that is selectively absorbed by the nucleoproteins of the chromosomes. We have so far obtained no evidence that methylcholanthrene will break chromosomes or bring about any chromosomal aberration in mice. All the mutations so far tested have been proven to be " point mutations." This phenomenon is of significance, since Burrows has shown that the carcinogens will cause chromosomes in growing root tips to lag on the spindle, thus indicating a mechanism of non-disjunction or other chromosomal aberrations to take place. The present author has obtained several possible disturbances in

sex ratios and in distorted mendelian ratios which may be later proven to be chromosomal, but so far all mutations induced by methylancholthrene in mice which have been tested have been proven to be point mutations. Finally, the mustard gas derivatives, the fourth group of powerful mutagens seem very effective in breaking chromosomes, producing embryonic disturbances very similar to the effects of X-rays, but apparently may have another action on mutagenesis, since they appear to affect cells even before there be any evidence of cell division (action therefore similar, but possibly different from that obtained with X-rays).

Carcinogenesis again is a relative rather than an absolute property. The mouse for some unknown reason is extremely sensitive to give rise to a variety of neoplastic lesions by any one of a great variety of chemicals. On the other hand, other species are extremely liable to great resistance to experimentally induced tumours. For example, the rhesus monkeys in New Haven have been injected with methylcholanthrene for 15 years without producing a single neoplastic tumour. One must conclude, therefore, that for this species methylcholanthrene is not a carcinogen, or at least, if it is, the evidence for such a conclusion is still not available. Even within the species, however, the property of carcinogenesis is not an absolute one. The end-result of the injection of a possible carcinogen, including the latent period, the survival time and the type of tumour induced or no tumour whatever depends upon the vehicle or solvent, the dose, the mode of introduction, the age of the experimental animal, etc. But even when all these environmental factors have been standardized, there is still great variability of results when mice of the various inbred strains are used. Greater variability of carcinogenesis is obtained when hybridization and selection are resorted to. Carcinogenesis in mice, therefore, is a variable characteristic. In the various strains of mice the estimation of carcinogenesis for methylcholanthrene differs from zero to 4.

It is also a very significant fact that the mouse is the only animate form in which the characteristics of mutagenesis and carcinogenesis can be compared in the same species. Briefly stated, therefore, the problem of comparing the properties of mutagenesis and carcinogenesis for any given group of physical and chemical agents is comparing and contrasting two extremely variable characteristics which differ between different species and even between different individuals of the same species. The problem is even more complex than this, as will be indicated in the following discussion.

Not all of the biological variants obtained in mice with methylcholanthrene are genetic. The present author has tested many of these effects obtained in the descendants of mice receiving methylcholanthrene, and is forced to the conclusion that many results are non-genetic. These variants are not considered to be non-genetic until they fail to reappear in the double backcross generation to the variant condition following an outcross to a totally unrelated strain. Another class of frequent occurrence is the somatic mosaic. These individuals are somatic composites of two genotypes for which no evidence of inheritance can be obtained. Another class of variants, such as the absence of one ovary alone or together with the congenital absence of one horn of the uterus is not genetic, and possibly may be explained in terms of specific inhibitions at particular or critical times of embryonic morphogenesis. It is a known fact that the carcinogens inhibit normal growth processes when injected into experimental animals, and there is

no reason why this process of inhibition may not apply to the development of the embryo at critical periods of differentiation. The somatic mosaic may be due to a chromosomal aberration rather than a point mutation in somatic differentiation, although the actual mechanism involved in their origin cannot be determined.

We have obtained, therefore, a multiplicity of biological effects when methylcholanthrene has been injected into a series of mice over many generations. Whether all these phenomena are due to the original methylcholanthrene or to one or many of the derived metabolites is still not known. Some of these effects are definite point mutations, some are non-genetic, some are non-genetic produced possibly by inhibitions of embryonic morphogenesis at critical periods, and other biological effects may have other mechanisms in their origin, such as abnormal distribution of chromosomes during morphogenesis.

But these mice whose ancestry has been injected with methylcholanthrene for many generations are not only giving rise to a wealth of biological effects, but also to a great variety of cancers. At least two genetic effects have been obtained in relation to induced tumours. The first genetic result was the production of germinal mutations which changed the rate at which fibrosarcomas appeared at the site of injection of methylcholanthrene (a decreased latent period). The second genetic effect was the production of spontaneous lesions involving the mucus-secreting cells of the gastric mucosa following the induction of this same gastric lesion from the subcutaneous injection of methylcholanthrene. The appearance of this specific type of pathological lesion was brought about by a mutation on the " brown tagged " chromosome. Several new sudden changes have appeared in the gastric lesion subline changing the latent period for the appearance of the lesion—a phenomenon that was obtained in the transplantation of adenocarcinomata of the mammary gland in mice and discussed in this same paper. Whether these new sudden changes are due to specific mutations may eventually be indicated.

The work on chemical carcinogenesis has provided much data of genetic interest. It appears now that the investigators of cancer can repay their debt to the investigators of general biology by reversing the process of borrowing observations in that field and to contribute information bearing on that science.

SUMMARY.

1. Sudden changes occur periodically in the transplantation of cancers in mice, which result in more malignant conditions by biological or genetic changes giving simpler and simpler mendelian ratios.

2. No two transplantable tumours ever have given the same mendelian ratio in the same series of F_2 individuals, even though the tumours were derived from the same mouse and were histologically indistinguishable.

3. A multiplicity of biological effects have been induced in mice by methylcholanthrene for which different biological mechanisms must be responsible.

4. As a general rule all mutagens are carcinogens and all carcinogens are mutagens, but exceptions to the rule must be kept in mind.

5. The mouse is the only animal where carcinogenesis and mutagenesis can be compared in the same species.

6. By accepting the concept that cancer arises from a process of somatic mutation, a pessimistic attitude for its eventual control is not indicated.

7. Somewhere in the completely resistant-to-cancer individual, produced or improved by the genetic process of selection, there must be a resistant-to-cancer mechanism.

8. When the resistant mechanism has been completely investigated that determines the biological state of resistance to cancer in all its points, no matter how great the insult, then this principle (possibly biochemical in nature) must control cancer in all species.

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AN EXPERIMENTAL STUDY OF THE INITIATING STAGE OF CARCINOGENESIS, AND A RE-EXAMINATION OF THE SOMATIC CELL MUTATION THEORY OF CANCER.

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THOUGH non-carcinogenic to normal skin, croton oil elicits tumours in skin previously treated with a carcinogen for an inadequate period (Berenblum, 1941). This procedure, with the modification of Mottram (1944), using only one single application of carcinogen prior to the croton oil treatment, served as the basis of a quantitative analysis of carcinogenic response (Berenblum and Shubik, 1947*b*). The results led to the establishment of the essential difference in mechanism between the preliminary "initiating process" and the subsequent "promoting process" of carcinogenesis. For reviews of the literature dealing with earlier work on the stages of carcinogenesis, and with the previous terminologies used, see Berenblum, 1944, 1947; Rusch, 1944; Berenblum and Shubik, 1947*a* and *b*.

From this analysis (Berenblum and Shubik, 1947*b*), it was concluded that the initiating process represents a sudden and irreversible change in a small minority of the cells of the treated area, giving rise to isolated "latent tumour cells," apparently indistinguishable morphologically from the surrounding non-neoplastic cells. The presence of these latent tumour cells is only demonstrable by subsequent promoting action, which converts them into morphological tumours. This promoting action is less specific than the initiating action, in that it can as readily be induced by croton oil as by continued applications of a true carcinogen. The irreversible nature of the initiating process was demonstrated by the fact that the number of tumours elicited by croton oil was as great after an interval of 20 weeks (between the carcinogen application and the commencement of croton oil treatment) as after an interval of only 3 days.

According to this new concept, the latent period of carcinogenesis is dependent on the efficacy of the promoting action, while the actual tumour yield is predetermined by the initiating action. This was borne out by comparative tests, using 1:2:5:6-dibenzanthracene, 3:4-benzpyrene, and 9:10-dimethyl-1:2-benzanthracene, as initiators, followed by croton oil treatment. It was found, as expected, that whereas the percentage of tumour-bearing animals differed in the three series, the latent periods were approximately the same.

Unfortunately, owing to considerations of solubilities, it was necessary, in these comparisons, to use a different concentration for each carcinogen. Consequently, more than one variable was involved in the experiment. While the results obtained provided an adequate basis for the general conclusions referred to above, it was felt, nevertheless, that unequivocal evidence could best be

provided by studying the effects of a series of graded concentrations of one and the same carcinogen as initiator. For this investigation, described below, 9:10-dimethyl-1:2-benzanthracene was chosen as initiator.

An additional purpose of this investigation was that the quantitative data thus obtained could, at the same time, serve as indirect evidence for or against the possibility of a " mutation-like change " for the initiating stage of carcinogenesis. However, for a re-assessment of the validity of the " somatic cell mutation theory of cancer " in the light of the dual-stage mechanism of carcinogenesis, a more direct experimental approach was required. Such an approach seemed possible from the following considerations.

Mustard gas is known to possess mutagenic properties when tested on Drosophila (Auerbach and Robson, 1946, 1947), and on Neurospora and *E. coli* (Tatum, 1947), so that, according to the somatic cell mutation theory, this compound should act as a carcinogenic initiator. Admittedly, mustard gas is a potent anti-carcinogenic agent (Berenblum, 1929), but this effect was shown to be confined to the later stages of carcinogenesis (Berenblum, 1929, 1931), and could thus be attributed to an anti-promoting action; thus, mustard gas should, theoretically, still be capable of initiating action. For a histological study of the effects of low concentrations of mustard gas on the mouse's skin, and the resemblance of the changes produced to those of a single massive dose of X-rays, see Fell and Allsopp, 1948.

Mustard gas was, therefore, tested for initiating action, under the standard experimental conditions, using croton oil as the promoting agent.

METHODS.

White female mice of the Swiss strain, originating from the Medical Research Council stock and since bred in this laboratory, were used for these experiments. The exclusive use of female mice eliminated the complication of skin irritation through fighting, the animals being kept in groups of 25 in large metal cages. The mice were maintained on an adequate mixed diet, with water *ad lib.* The experimental areas of skin, in the inter-scapular regions, were clipped periodically with fine scissors for removal of hair. All test solutions were applied with a glass rod, and colourless, non-fluorescent, liquid paraffin (" liquid Petrolatum ") was used as solvent throughout, for standardization of response (Berenblum and Schoental, 1947; Berenblum and Shubik, 1947*a*).

The main departure from the technique of the previous experiments was that instead of merely noting the numbers of tumour-bearing mice, the individual tumours were also recorded. The method adopted was to number each mouse by ear punching as soon as a tumour appeared, and to chart that tumour, and all subsequent ones, at fortnightly intervals throughout the experiment. Thus, the results are expressed in terms both of total numbers of tumours induced and of numbers of tumour-bearing animals, the former being also expressed in a corrected form, to exclude those " tumours " which regressed within 2 weeks of their appearance, whose neoplastic nature was obviously in doubt, and never confirmed.

The tumours produced were all papillomas in the first instance, judged by naked-eye evidence, and in many cases confirmed by histological examination after death. These papillomas appeared to behave very much like those induced

by repeated painting with carcinogens, with a tendency to grow in size, and, in many cases, to become carcinomas, but also with a tendency for some of the papillomas to regress after a period of progressive growth. There was only one exception, in which the malignant tumour induced had the histological character of a sarcoma.

EXPERIMENTAL.

Comparison of graded concentrations of initiator.

Four groups of 100 mice each, were used for this investigation. These received a single painting of 9:10-dimethyl-1:2-benzanthracene in liquid paraffin, to serve as initiator. Four weeks later, the same area of skin was submitted to twice-weekly applications of croton oil in liquid paraffin for 25 weeks, for promoting action.

The concentration of initiator solution varied in the 4 groups as follows :

Group AG, 0·06 per cent ; Group AA, 0·17 per cent ; Group AB, 0·5 per cent ; Group AC, 1·5 per cent.

The croton oil solution used was 5 per cent (except that a 10 per cent solution was used for 25 mice of each group for the first 7 weeks. Since the results obtained were, in each case, essentially the same as in the remaining 75 mice, the figures were combined). A control group of 25 mice which received a single application of 9:10-dimethyl-1:2-benzanthracene, without subsequent croton oil treatment, failed to yield any tumour.

TABLE I.—*The Potency of* 9:10-*Dimethyl*-1:2-*benzanthracene as Initiator, Tested in Different Concentrations, when Applied Once Only to the Mouse's Skin, Followed by Repeated Croton Oil Treatment.*

Series.	Concentration of carcinogen. %.	Latent period (weeks).	Number of mice with tumours.	Total number of tumours.	Average number of tumours per tumour-bearing animal.
AG	0·06	14·4	9	10	1·1
AA	0·17	12·1	25	61	2·4
AB	0·5	10·6	43	118	2·7
AC	1·5	11·8	63	248	3·9

Duration of experiment : 29 weeks (i.e. 25 weeks from commencement of croton oil treatment).
Number of animals : 100 per group.

The results obtained are summarized in Table I, showing : (1) the average latent period per group (estimated on the basis of the appearance of the first tumour per animal) ; (2) the numbers of tumour-bearing mice ; (3) the total numbers of individual tumours, and (4) the average number of tumours per tumour-bearing animal.

It will be noted that, with the ascending increase in the concentration of initiator, there was a progressive increase in the tumour yield, both as expressed in terms of tumour-bearing animals per group, and, more strikingly, in terms of individual tumours produced ; while the average number of tumours per tumour-bearing animal also increased (from 1·1, for the lowest concentration, to 3·9 for

the highest). Nevertheless, the average latent periods showed no significant variation in the 4 groups. These results may be taken as a clear and striking confirmation of the concept (Berenblum and Shubik, 1947*b*) that the tumour incidence is directly dependent on the initiating action, while the latent period is a function of the promoting action.

TABLE II.—*A Comparison of Ratios of the Results Obtained (Table I).*

Series.	Ratios of: Concentration of carcinogen.	Numbers of tumour-bearing mice.	Total numbers of tumours.	Total numbers of tumours, less early regressions.
AG	1	1·0	1·0	1·0
AA	3	2·8	6·1	6·6
AB	9	4·8	11·8	12·4
AC	27	7·0	24·8	26·5

When the results are compared in terms of ratios (Table II), it becomes apparent that the actual tumour yield approximately bears a direct 1:1 relation to the concentration of the initiator (thus, with a 27-fold increase in concentration of initiator, there was a 26·5-fold increase in tumour yield). Its significance in relation to the somatic cell mutation theory of cancer will be discussed below.

Tests of mustard gas for initiating action.

Two groups of 25 mice were used for this investigation, one receiving a single, and the other 3 weekly, applications of a 0·1 per cent solution of mustard gas in liquid paraffin. Both groups were then left untreated for 3 weeks, and thereafter the experimental area of the skin was painted twice weekly for 25 weeks with a 5 per cent solution of croton oil in liquid paraffin.

Except for 3 small nodules, at first believed to be early papillomas, which regressed within 1–4 weeks, no tumours appeared in either group.

DISCUSSION.

It was previously shown (Berenblum and Shubik, 1947*b*) that by varying the nature and concentration of carcinogen as initiator (applied once only), followed by repeated croton oil applications, different tumour yields were obtained, though the average latent periods for the different groups remained approximately the same. From these results, and from other evidence brought forward, it was concluded that the ultimate tumour yield in carcinogenesis is pre-determined by an irreversible initiating process, while the latent period of carcinogenesis, i.e. the speed with which the induced "latent tumour cells" are converted into morphological tumours, is a function of the subsequent promoting process.

The idea of the possible existence of "latent tumour cells" requiring additional stimulation, i.e. promoting action, for their conversion into morphological tumours, arose from the work of Friedewald and Rous (1944) on the disappearance and reappearance of carcinogenically-induced papillomas in rabbits. The experiments on mice with croton oil (Berenblum and Shubik, 1947*b*) provided the quantitative data for the establishment of the two-stage mechanism of carcinogenesis, with "latent tumour cells" as the product of the first, or

initiating, stage. A similar belief in the existence of " latent tumour cells " as the basis of tumour production, has also been expressed by Fischer (1937), as a consequence of his studies on tumour development following the repeated transplantation of normal mammary tissue in series, in mice.

An interesting confirmation of the two-stage mechanism of carcinogenesis has recently become available (Hall, 1948), from a follow-up of the work of Bielschowsky (1945, 1947) on tumour production in the thyroid gland by the combined action of 2-acetylaminofluorene and goitrogenic agents. Hall studied the effect of the goitrogenic agents before and after a period of treatment with acetylaminofluorene, and found that augmentation of tumour induction only occurred when these were administered after the acetylaminofluorene. The effect was still observed when there was a long interval between the two treatments. The results suggest that the acetylaminofluorene acted as initiator, and the goitrogenic agent as promoter, in an analogous fashion to the carcinogen and the croton oil, respectively, in the case of skin experiments.

In the present investigation, one and the same carcinogen was applied to the mouse's skin, once only in different (graded) concentrations, to serve as initiator, and then followed by repeated applications of croton oil. Expressed in ratios, the concentrations of initiator (9:10-dimethyl-1:2-benzanthracene in liquid paraffin) in the four experimental groups were 1:3:9:27, and the total yields of tumours for the corresponding groups were in the ratio of 1:6·6:12·4:26·5 (Table II). Yet the average latent periods for these 4 groups were closely similar, namely, 14·4, 12·1, 10·6, and 11·8 weeks, respectively. Not only was there a progressive increase in the total number of tumours, with increase in concentration of initiator, and also in the number of tumour-bearing animals, but the actual number of tumours per tumour-bearing animal rose progressively, from an average of 1·1, in the lowest concentration, to 3·9 in the highest.

These results provide a striking confirmation of the concept outlined above concerning the two-stage mechanism of carcinogenesis.

The evidence that the initiating process of carcinogenesis is sudden and irreversible would seem to suggest a " mutation-like " change, and the indication that the change affects very few of the cells in the treated area (possibly even a single cell per locus) also supports this supposition. It became necessary, therefore, to re-examine the somatic cell mutation theory of cancer in the light of this new concept.

This theory, first dealt with at length by Bauer (1928), and reviewed by Ludford (1930), Lockhart-Mummery (1934), and many others, considers neoplasia to be the consequence of a changed gene. It can, by its very nature, only be submitted to verification of an indirect kind (Haldane, 1934), since the only proof of a mutation is that achieved by crossing the mutated cell with a normal cell. In the case of a somatic cell, which cannot conjugate, this is manifestly impossible. Many different experimental approaches have, however, been devised in recent years to obtain, at least, indirect evidence in support of the theory, and these may conveniently be considered under the following headings :

1. *Induction, by known carcinogenic agents, of mutations in lower organisms.*

The early studies in this field were concerned with X-rays and ultraviolet radiation, the literature of which is well reviewed by Muller (1941). Although most of this work was not directly concerned with correlating carcinogenicity

and mutagenic potency, the fact that an apparent association, in the case of physical agencies, did exist, was widely accepted as strong evidence in support of the somatic cell mutation theory of cancer.

The discovery of chemical mutagens (e.g. mustard gas) by Auerbach and Robson (1946, 1947), helped to extend the field of enquiry, and made possible detailed comparisons between carcinogenic and mutagenic activity of various compounds, especially among the polycyclic hydrocarbons.

Tatum (1947), using neurospora as indicator, studied various types of mutations induced with 20-methylcholanthrene, but, while reviewing the somatic cell mutation theory, he did not make quantitative correlations between the two properties possessed by this compound. Using various carcinogenic and non-carcinogenic hydrocarbons in the form of aerosols, tested on drosophila, Demerec (1948) was able to find some correlation between carcinogenicity and the power to produce mutation, though the correlation was not absolute.

Demerec and Latarjet (1946) had previously tested X-rays and ultraviolet radiations on bacteria, giving precise data in relation to mutagenic and lethal power. More recently, using a wide range of both water-insoluble (colloidal dispersions) and water-soluble carcinogenic and non-carcinogenic compounds, tested under similar conditions to those of Demerec (1948) and studied on a quantitative basis, Latarjet (1948) failed to find any correlation between mutagenic and carcinogenic potencies.

Briefly, therefore, the apparent correlation between mutagenic and carcinogenic activities, as noted in the case of physical agencies, has not been confirmed when extended to chemical agents, and can no longer be used as strong support in favour of the somatic cell mutation theory of cancer.

2. *Genetic analysis of tumour transplantation phenomena in pure strain mice.*

The genetic control of tumour transplantation in pure strain mice has been investigated in some detail, and the earlier work in this field is well summarized by Little (1941). It has been shown that a number of genes control the behaviour of certain tumours, and this approach has been extended further by Gorer (1937, 1947), showing that these genes probably determine their antigenic properties. Also, according to Strong (1926), Bittner (1931) and others, the observed sudden change in the transplantation properties of tumours could be explained on the basis of the occurrence of mutations. The comparison between transplantation properties of normal and tumour tissues, originally suggested by Little (1941), was undertaken by Furth, Boon and Kaliss (1944), who made such comparisons between certain transplantable tumours and leukaemias against normal spleen, and found the transplantation behaviour to be different in the two classes of tissues, thus pointing to a difference in their genetic make-up. From this they concluded that a somatic mutation would be a more likely explanation of the changeover from the normal to the neoplastic state than any type of "differentiation."

It must be remembered, however, that differences in transplantation behaviour involve many so-far unexplained anomalies, some of which have been forcefully brought out by the work of Greene (1944), and that analogies between the genetics of transplantation (with all the complications of adaptability in another host) and that of tumour induction (where the autologous nature of the

tumour cells is self-evident) may be more complex than is generally supposed. This type of evidence cannot, therefore, be considered as more than suggestive in support of the somatic cell mutation theory.

3. *Induction of germinal mutations in mice by means of carcinogens.*

The work of Strong (1945) on the development of germinal mutations in mice injected with methylcholanthrene, led him to suggest that somatic mutations might well be engendered by the same agency. Carr (1947) has also claimed to have produced such germinal mutations with 1:2:5:6-dibenzanthracene. The validity of the results have been questioned (Heston, 1948).

So far, the number of germinal mutations attributed to carcinogenic hydrocarbons has been very small, and further work along these lines is clearly needed, in order to determine whether carcinogenic hydrocarbons are, indeed, more effective than other compounds in inducing germinal mutations. Until this is known, it is difficult to decide how far these results are significant as evidence in favour of the somatic cell mutation theory.

4. *Quantitative interpretation of carcinogenic experiments.*

Several attempts have been made to evaluate quantitatively some of the more reliable carcinogenic data, to determine whether or not such data would be in keeping with a sudden irreversible change of a mutation-like nature.

In the investigation of Dunning, Curtis and Wood (1940), the production of sarcomas in rats, by subcutaneous injection of 3:4-benzpyrene, was submitted to quantitative analysis. The concentration of carcinogen and the volume of fluid injected, both of which were variables, were correlated with the number of tumours induced and with the latent period of tumour induction. It was found that increasing the number of foci injected did increase the number of tumours induced, whereas increasing the volume of fluid injected in one locus did not. In view of the variables involved, and for other reasons to be discussed later, these results do not lend themselves to an exact interpretation of a single response to a single stimulus.

Charles and Luce-Clausen (1942) analysed data of a mouse skin carcinogenesis experiment, using continued painting with 3:4-benzpyrene, to determine whether or not this was in keeping with the concept of the somatic cell mutation theory. In their introduction, they make the primary assumption that if a mutation were the basis of carcinogenesis, it must, of necessity, be of a recessive nature, thus postulating two successive mutations to bring to light an actual tumour. There is, however, no valid reason for this assumption, which is, in fact, at variance with the theory of dominancy, as proposed by Fisher (1930).

In all these previous attempts at analysing carcinogenic experiments, in which the carcinogen is allowed to act continuously, there is the inevitable complication arising from the fact that, of the two stages of carcinogenesis, only one—the initiating stage—could possibly be ascribed to a mutation, since this is the stage which involves a sudden and irreversible change, possibly affecting one cell only. A true quantitative analysis, bearing on the mutation theory, can only be acquired by examining the carcinogen in an uncomplicated light, i.e. as initiator only, and, if possible, on one occasion only in this role.

In the present investigation, the correlation of concentration of carcinogen, as initiator, with the total number of tumours induced, has revealed approximately a direct 1:1 ratio. This could be taken as being in keeping with the somatic cell mutation theory.

However, the other investigation, described above, does not lend support to this theory. As already mentioned, mustard gas is a potent mutagenic agent on drosophila (Auerbach and Robson, 1946, 1947). Since its anti-carcinogenic properties (Berenblum, 1929, 1931) can undoubtedly be attributed to anti-promoting action, it was to be expected, according to the somatic cell mutation theory, that when allowed to act once only on mouse's skin, followed by repeated croton oil treatment, tumours should have arisen, the mustard gas acting as an initiator by virtue of its mutagenic properties. When tested, this was found not to be the case.

In view of this negative finding, and in the light of the highly conflicting evidence of recent experiment in an attempt to adduce more indirect support of the theory, it becomes necessary to re-assess the validity of the theory.

Basically, neither proof nor disproof of the somatic cell mutation theory of cancer is possible (Haldane, 1934), as referred to above. The accumulating indirect evidence would seem to run counter to the theory instead of supporting it. Of the evidence presented in the present communication, the first experiment is consistent with the theory, while the second is strongly against it.

The force of past reasoning in support of the theory has rested largely on the assumption that, given an irreversible change as the basis of carcinogenesis, the only known biological phenomenon to explain this would be a mutation. However, a closer examination of other common biological phenomena instantly reveals that this is not so. For example, in the course of the development of the embryo, the divergent differentiation that ultimately results in the irreversible cell types, e.g. into epithelial, nerve, and muscle cells, etc., represents just such a non-mutational irreversible process. Moreover, from the early work of Cohnheim, it is possible that aberrant embryonic differentiation may occur, though this probably provides no more than a partial explanation of certain rare and very specialized groups of tumours.

The substitution of a concept of non-mutational " aberrant differentiation," divorced from Cohnheim's all-embracing embryological implications, in the place of a somatic mutation mechanism, to account for the initiating process of carcinogenesis, clearly deserves further consideration. It is tempting to draw a parallel, from the knowledge of embryological development, between the " organizer " and the " latent tumour cell," on the one hand, and between the " evocator " and the " carcinogenic promoting agent " on the other. In our present state of knowledge, such a hypothesis would, admittedly, be a dangerous over-simplification ; nevertheless, as a guide for further investigation, the analogy may possibly serve a useful purpose. On the question of the mechanism of non-mutational, hereditable transmission of new characters, the action of viruses, particularly the example of the milk-factor in the development of mammary carcinoma in mice, Bittner, 1936, 1939, and the recent work of Sonneborn (1948), on the transmission of antigenic and other differences in genetically identical paramecium cells, should also be taken into account.

In conclusion, whatever interpretation is adopted as a base-line for research, the recognition that carcinogenesis is at least a two-stage process, should in-

variably be borne in mind. Any basic theory that overlooks this must, of necessity, be incomplete.

SUMMARY.

1. With the standard experimental technique, using a single application of a carcinogen followed by repeated paintings of the same area of skin with croton oil, a quantitative correlation has been made between the concentration of the carcinogen (initiating agent) and the total number of tumours induced.

2. It was found that there exists approximately a direct 1:1 ratio between the two.

3. The known mutagenic agent—mustard gas—was investigated under the conditions of the standard experiment, as initiating agent, and found to be negative.

4. The results are discussed in relation to the somatic cell mutation theory of cancer.

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THE INDUCTION OF TUMOURS WITH NITROGEN MUSTARDS.

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RECENT experience has shown that many of the growth-inhibiting agents used in the palliative treatment of cancer are carcinogenic. They also cause specific damage to cell nuclei and chromosomes and are able to induce mutations. The association of these biological effects of (1) growth inhibition, (2) chromosome damage, (3) production of mutations and (4) induction of cancer suggests that they may have a common fundamental biochemical mechanism. If the induction of cancer is indeed a somatic mutation, then cancer induction might be included as a special mutation. The fact that X-rays can produce cancer in man was published in 1902 (Frieben, 1902), seven years after Röntgen's discovery of X-rays. Müller (1928) found that X-rays are mutagenic, and later Mather and Stone (1933) and Koller (1934) described the chromosome damage following irradiation. The idea that cancer arises as a somatic mutation was supported by the demonstration of an increased incidence of mutations occurring in mice treated with chemical carcinogens. This was shown with methylcholanthrene by Strong (1945) and with 1:2:5:6-dibenzanthracene by Carr (1947). The carcinogenic hydrocarbons inhibit the growth of animals and the establishment and growth of transplanted tumours (Haddow, Scott and Scott, 1937). They are able to cause breaking of chromosomes in tumour cells (Koller, personal communication). One of the most potent carcinogenic hydrocarbons, 9:10-dimethyl-1:2-benzanthracene, has been used with some temporary success in the treatment of lymphocytic leukaemia (Engelbreth-Holm and Stamer, 1947).

In the treatment of leukaemia (Paterson, ApThomas, Haddow and Watkinson, 1946), urethane has been used as an alternative to radio-therapy. This drug also induces cancer of the lung in mice (Nettleship and Henshaw, 1943) and inhibits mitosis (Dustin, 1947). Derivatives of 4-dimethylaminostilbene also have this dual action. These compounds were first found to be growth inhibitors, and later found to be potent carcinogens (Haddow, Harris, Kon and Roe, 1948).

The vesicants, mustard gas (di(2-chloroethyl)sulphide) and nitrogen mustards (e.g. methyl di(2-chloroethyl)amine, HN2 and tri(2-chloroethyl)amine HN3) cause increased stickiness of chromosomes and rupture of chromosomes (Darlington and Koller, 1947; Boyland, Clegg, Koller, Rhoden and Warwick, 1948). They also produce mutations, as was shown in drosophila by Auerbach and Robson (1947) and in neurospora by Horowitz, Houlahan, Hungate and Wright (1946) and by Tatum (1947). The numerous other radiomimetic actions of the nitrogen mustards, which have been discussed elsewhere (Boyland, 1948), show the remarkable similarity in action of vesicants and radiations. Mustard gas was shown to have an anticarcinogenic action by Berenblum (1931), and this antagonistic action may be similar to the antagonism of weak carcinogens to potent carcinogens described by Lacassagne, Buu-Hoï and Cagniant (1944). By analogy with previous findings the nitrogen mustards might be expected to have carcinogenic action, and the actual induction of tumours by these substances is described in the present paper.

EXPERIMENTAL.

Two groups each of 20 stock mice were given weekly subcutaneous injections of 10 ml. per kg. body-weight of freshly made aqueous solutions of nitrogen mustard. The first group was given methyl di(2-chloroethyl)amine hydrochloride (HN2) in a concentration of 1 mg. per 10 ml., so that it received 1·0 mg. per kg. body-weight at weekly intervals for 50 weeks. The second group received the same dose of tri(2-chloroethyl)amine hydrochloride (HN3) for 10 weeks, after which time only 4 mice remained alive and injections were stopped.

Post-mortem findings of the mice which died within a week of an injection usually showed congestion of the alimentary tract or lungs. With the doses used no nervous symptoms were seen. All the coloured mice showed the effect of greying of hair (Boyland *et al.*, 1948) similar to that produced by X-rays (Hance and Murphy, 1926) or by subcutaneously implanted plutonium (Prosser, Painter, Lisco, Brues, Jacobson and Swift, 1947). The de-pigmented area of fur usually became visible at the site of injection after 2 or 3 weeks. Other experiments have shown that intradermal injection of the nitrogen mustard is more effective than subcutaneous injection in producing this effect on hair colour.

Delayed effects of treatment.

Differential blood counts were made on the surviving mice 15 months after the beginning of the injections. Three of the HN2 series (No. 16, 18 and 19) and three of the HN3 series (No. 38, 39 and 40) were leucopenic. The blood picture was abnormal (Table I) in showing a deficiency of lymphocytes. Cameron, Courtice and Jones (1947) found that injection of HN2 into rabbits and dogs caused neutropenia and lymphocytopenia. The present results indicate that the repeated injection of nitrogen mustards induces a chronic lymphocytopenia in a proportion of treated mice.

One mouse (No. 10) which died 148 days after beginning of treatment with HN2 showed (1) slight proliferation of the bronchiolar epithelium, (2) mobilization of lymphocytes round ducts and blood vessels of the liver, and (3) aggregation of lymphocytes around bronchioles and blood vessels of the lung.

TABLE I.—*Blood Counts of Mice made 13 Months after Commencement of Treatment.*

Mice No. 14–20 treated with HN2.
Mice No. 37–40 treated with HN3.

Mouse No.	R.B.C. count in millions.	Hb % (Haldane).	Total leucocytes.	Differential count of per cent of total.				
				Neutrophil polymorphs.	Lymphocytes.	Monocytes.	Eosinophils.	Unclassified immature cells.
14	6·4	44	46,400	66	31	1	..	2
15	7·3	72	46,400	70	24	1	..	5
16	3·3	38	10,000	30	66	..	..	4
17	7·3	73	8,000	35	62	3	..	..
18	9·2	88	16,000	45	50	5	..	..
19	10·1	96	12,600	44	52	3	1	..
20	10·6	103	14,600	60	38	2	..	..
37	10·1	110	18,400	41	53	1	..	5
38	11·9	110	9,400	58	41	..	..	1
39	10·5	108	6,000	32	67	1	..	..
40	9·8	94	5,600	39	69	2	..	..
Untreated (mean of 20 mixed stock mice)	10·2	103	19,000	17	79	2	1	1

The post-mortem findings in mice which survived more than 280 days are shown in Tables II and III. Of the total of 14 mice, 10 had tumours, including lung carcinomas and adenomas in 8, lymphosarcomas in 2, a uterine fibromyoma and a sarcoma at the site of injection. Of the mice free of neoplasms 2 had lymphocytic infiltration of the liver and lung and 2 had abnormal proliferation of the bronchiolar epithelium. Most of the mice with lung tumours or lymphosarcomas in the liver had enlarged lymph nodes.

A group of 40 untreated mice from the same source as the experimental mice were killed when between 14 and 18 months of age. In this series 6 mice had adenomas of the lung, 2 hepatomas and 3 had enlarged lymph nodes.

The mice with lymphosarcomas had greatly enlarged livers (Fig. 1), in which the normal structure was destroyed (Fig. 2), the malignant cells showing numerous mitoses (Fig. 3).

The lung tumours were for the most part larger and more malignant (Fig. 4 and 5) than those occurring spontaneously in mice. Many of these lung tumours

DESCRIPTION OF PLATES.

FIG. 1.—Mouse 12 (decapitated). Treated for 347 days with HN2. This rodent developed a lymphosarcoma in the liver, together with enlarged thymus and lymph nodes. Macroscopic lesions can be seen in liver. × 1¼.

FIG. 2.—Lymphosarcoma; section of liver of mouse seen in Fig. 1. × 105.

FIG. 3.—Mouse 40. Subcutaneous spindle-celled sarcoma which developed at site of injection with HN3. × 210.

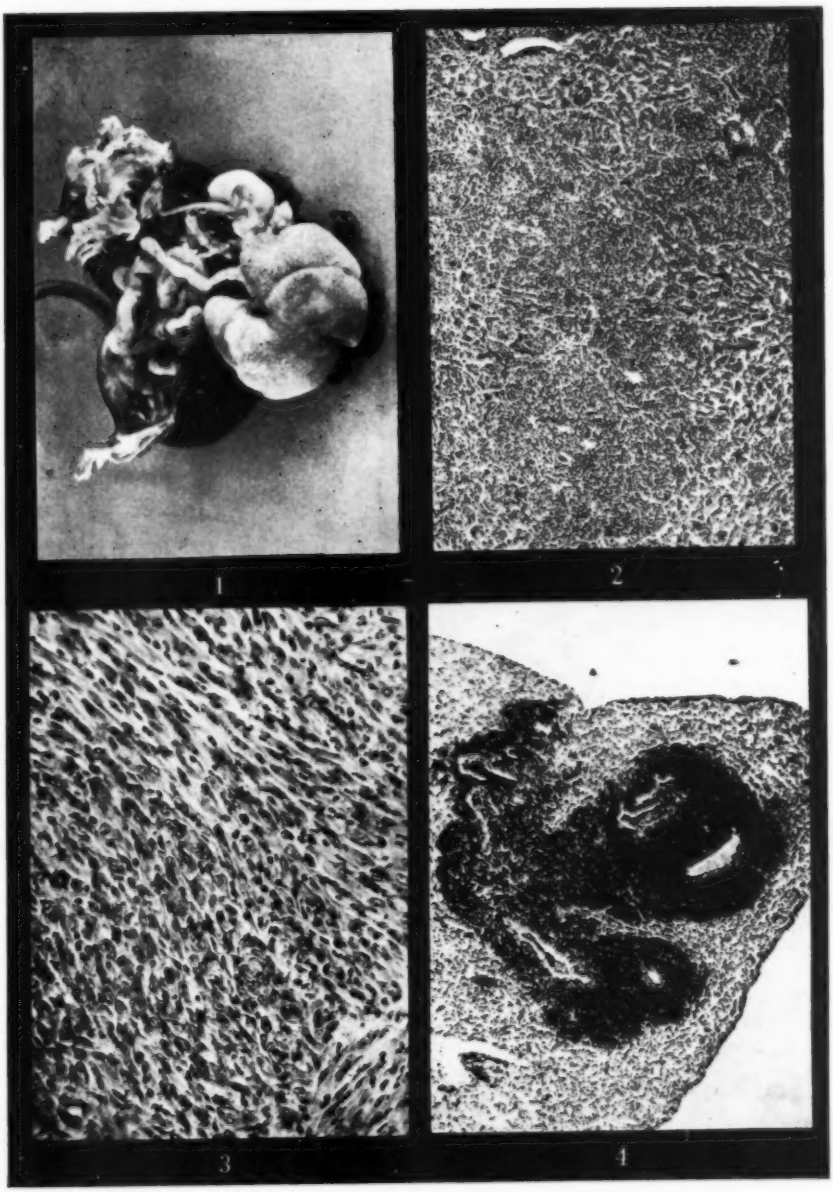
FIG. 4.—Mouse 15. Showing dense aggregation of lymphocytes around bronchioles in lung following treatment with HN2. × 58.

FIG. 5.—Mouse 17. Adenocarcinoma of lung following treatment with HN2. × 105.

FIG. 6.—Mouse 38. Carcinoma of lung following treatment with HN3. Tumour cells are seen invading the alveolar tissue of the lung. × 210.

FIG. 7.—Same as Fig. 6. Showing another field in the same section, in which the lumen of a bronchiole is occluded by tumour cells. × 210.

FIG. 8.—Mouse 19. Treated with HN2. Carcinoma of lung. × 210.



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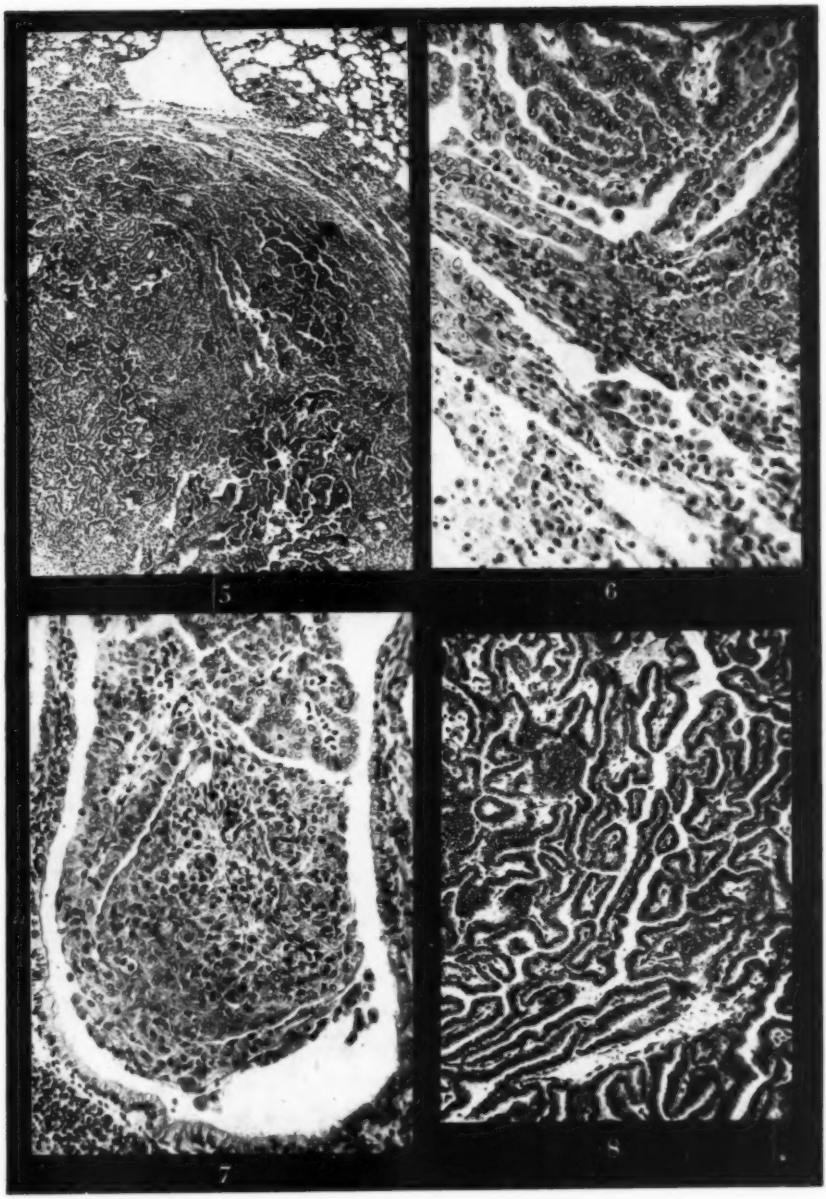

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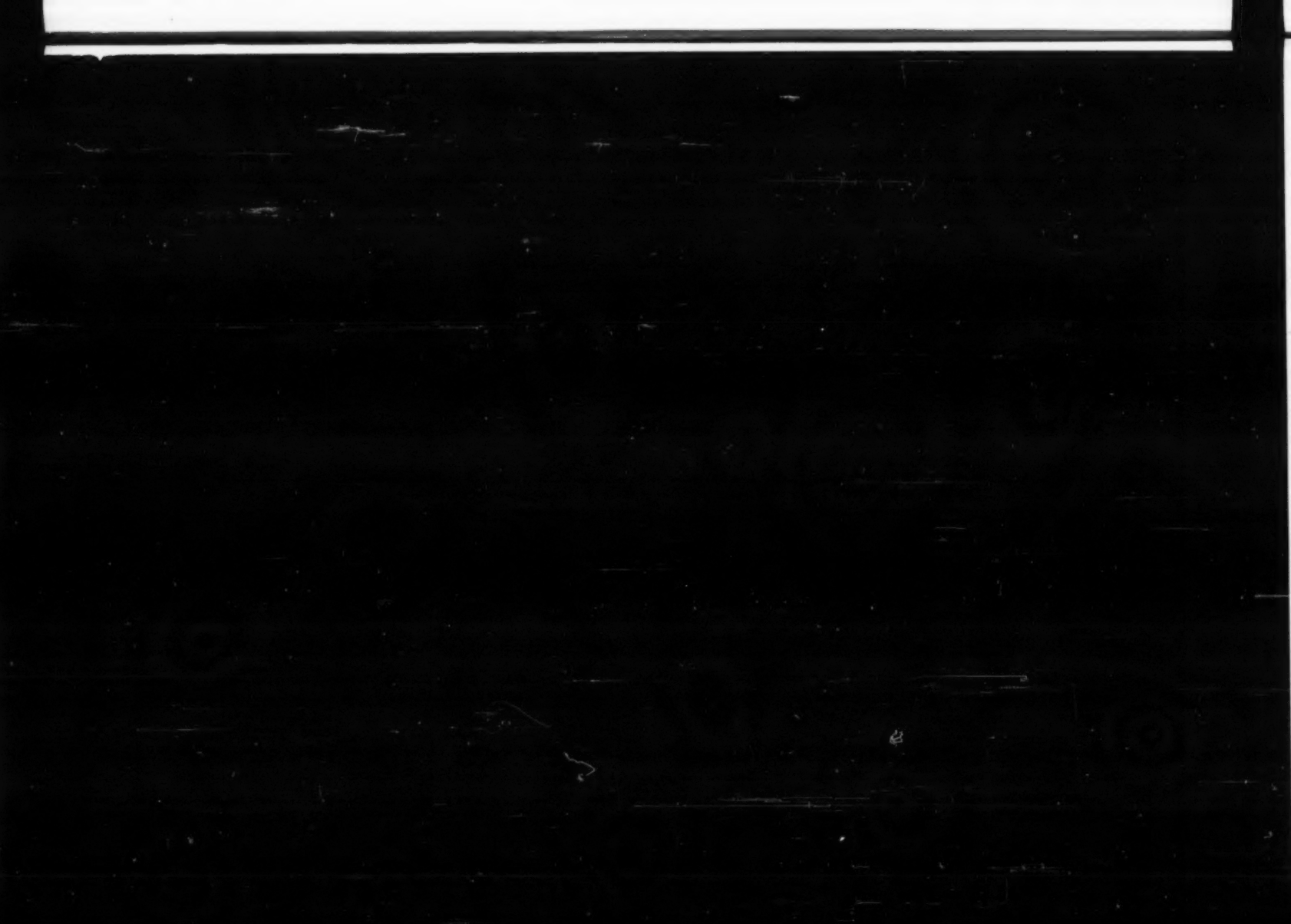

TABLE II.—*Mice Injected with Weekly Doses of* 1·0 *mg./kg. of Methyl Di-(2-chloroethyl)amine Hydrochloride (HN*2*) till Death or for* 50 *Weeks.*

Mouse No.	Survival time (days).	Post-mortem findings.
10	148	Proliferation of bronchiolar epithelium with aggregation of lymphocytes round ducts and vessels of liver.
11	284	*Lung*, adenoma.
12	347	*Liver*, lymphosarcoma with dense lymphocytic infiltration and cell degeneration. *Lymph nodes* and *thymus* greatly enlarged.
13	367	*Lung*, proliferation of bronchioles; early bronchogenic tumours and thickening of interalveolar septa.
14	398	*Liver*, slight lymphocytic infiltration. *Lung*, abscess, destruction of alveoli, dense lymphocytic infiltration with polymorphs.
15	399	*Liver*, marked lymphocytic infiltration. *Lung*, thickening of interalveolar septa.
16	550	*Uterus* fibromyoma. *Lung*, proliferation of bronchiolar epithelium with fibrosis and internal infiltration of lymphocytes in blood vessel.
17	569	*Lung*, carcinoma accompanied by dense lymphocytic infiltration. *Liver*, slight lymphocytic infiltration accompanied by degeneration of liver cells. *Spleen*, *thymus* and *lymph nodes* enlarged.
18	569	*Lung*, *carcinoma* with aggregation of lymphocytes around blood vessels and bronchioles. *Prostate*, hypertrophy of prostatic epithelium, not so pronounced as in No. 40. *Lymph nodes* enlarged.
19	575	*Lung*, carcinoma.
20	580	*Liver*, marked lymphocytic infiltration around blood vessels and ducts.

TABLE III.—*Mice Injected with* 10 *Doses of* 1·0 *mg./kg. of Tri-(2-chloroethyl)amine Hydrochloride (HN*3*) which Survived More than* 54 *Days.*

Mouse No.	Survival time (days).	Post-mortem findings.
37	548	*Lung* adenoma with lymphocytic infiltration.
38	552	*Lung*, bronchogenic carcinoma infiltrating alveolar tissue.
39	560	*Lung*, bronchiolar proliferation. *Kidney*, glomerulonephritis.
40	567	*Lung*, carcinoma. Spindle-celled *sarcoma* at site of injection. *Prostate*, pronounced hypertrophy of epithelium in anterior lobe. *Liver*, lymphocytes around duct. *Thymus*, *lymph nodes* and *parotid* enlarged.

were invading the normal tissue, and in many cases the lungs of these mice had aggregations of lymphocytes.

The spindle-celled sarcoma at the site of injection of mouse No. 40 was still small when the mouse was killed, and was immediately below the area of depigmented skin where injections had been made.

DISCUSSION.

The results of the experiments suggest that the nitrogen mustards have a slow carcinogenic action. As only 14 mice survived to an age when tumours occur the experiments are being extended in mice of pure strains. The incidence of lung tumours and lymphosarcomas was higher than would be expected to

occur spontaneously, and the tumours were larger and more malignant than those generally encountered in untreated mice.

The carcinogenic action of the nitrogen mustards is very slow, the earliest lung tumour and lymphosarcoma being seen at 284 and 347 days respectively. Berenblum and Shubik (1947) have shown that carcinogenesis can be divided into an initial stage in which cells are irreversibly changed into latent tumour cells, and a development stage which can be brought about by cocarcinogens such as croton oil. The incidence of tumours expressed as quantal response is probably dependent on the initiating action, but the rate of appearance of the tumours depends upon cocarcinogenic action.

The present authors consider that the initial irreversible conversion to latent tumour cells is the change which is associated with chromosome damage and mutations. The nitrogen mustards are possibly active in initiating the carcinogenic change but poor in developing action. In order to test this hypothesis experiments are in hand in which mice are being treated with nitrogen mustard and croton oil.

A possible explanation of the association between inhibition of growth and carcinogenic power is that the inhibition is due to interference with cell division in growing tissues. The drugs under discussion reduce the rate of growth by impeding mitosis. This interference with mitosis is likely to increase the chances of a somatic mutation from normal to malignant cells occurring. The somatic mutation is possibly a relatively rare event, the occurrence of which is more probable when the difficulties of cell division are increased either by radiations or by chemical agents.

SUMMARY.

Mice were given weekly subcutaneous injections of nitrogen mustards. Of 14 mice which survived more than 250 days, 10 had tumours including lung tumours (8), lymphosarcomas (2), a uterine fibromyoma and a spindle-celled sarcoma at site of injection.

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CARCINOGENIC ACTIVITY OF LIPOID SUBSTANCES.

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THE investigation of carcinogenic substances which have been obtained from human tissues (Kleinenberg, Neufach and Shabad, 1940) and from other biological sources has been the subject of earlier publications from this Institute (Hieger, 1940, 1941, 1946, 1947), reporting that :

1. A carcinogen is present in extracts from human subjects who have died of cancer *or* of other causes.
2. The carcinogen is found in the unsaponifiable part of a number of tissues, such as liver, lung, kidney and muscle.
3. It is found in the cholesterol-rich part of the unsaponifiable fraction, which contains *c.* 85 per cent of the digitonin precipitable sterol and is referred to below as "Fraction A" or "Stage A," or "— 10° fraction," since it crystallizes out at — 10° when the unsaponifiable fraction of the tissue (doubly saponified) is crystallized from methyl alcohol.
4. Commercial cholesterol containing 95–98 per cent of the pure sterol is carcinogenic, and its potency is of the same order as that of the active fractions from human tissues.

With very few exceptions the carcinogen in biological material is slow acting (Table V), by comparison with synthetic carcinogens. Hence the tests on animals are lengthy.

Since some of the experiments described here were in progress when the papers referred to above were published and others have not before been described in detail, a certain amount of repetition is unavoidable.

1. *Technique used in these experiments for testing for carcinogenic activity.*

Unless otherwise stated, the substance is injected subcutem. (15 per cent solution in lard) into mice of C57 strain or of mixed commercial stock. The injection of 0·1–0·4 ml. is repeated fortnightly until a reservoir remains under the skin ; injections are suspended as long as a nodule can be detected by touch. All tumours referred to are spindle-cell sarcomas arising at the site of injection (Fig. 1–8). Precautions were taken to prevent contamination by carcinogens from outside (Hieger, 1946).

2. *Control experiment on reagents used in the saponification of tissue by potassium hydroxide and alcohol.*

Since the great majority of the carcinogenic fractions had undergone saponification at least once, control experiments on the process itself acting on non-carcinogenic tissue components were made. The saponification conditions were described in detail (Hieger, 1946) ; about 1500 g. tissue is refluxed on the water bath with 400 g. KOH and 500 c.c. distilled alcohol for 2–3 hours and then extracted several times with ether. Carcinogens might be formed during saponification from precursors in the tissues or from the reagents themselves.

In the earlier experiments the alcohol was industrial spirit (the manufacturers state that it consists of ethyl and methyl alcohols in the ratio of 19:1 with 5 per cent water), which in separate experiments was found to darken rapidly on refluxing with KOH owing to resinous condensation of aldehyde. Acetaldehyde was therefore refluxed with KOH and spirit under the same conditions as those of tissue saponification. The resinous product was extracted from the saponification mixture with ether and injected into mice in the usual way. In another experiment the resinous product before injection into mice was mixed with cholesterol, which was intended to act as a carrier, in order to imitate rather more closely the products and conditions after tissue saponification (Experiments 1 and 2). All the tests on resin gave negative results.

Although in the earlier experiments spirit was used for the saponification, a number of unsaponifiable fractions made from adrenals, cancer liver and non-cancer liver (see note to experiment 3*a* for a definition of these terms) were found to be non-tumour-producing, which suggests that carcinogens or precursors are

DESCRIPTION OF PLATES.

FIG. 1.—Sarcoma in a C57 ♂ mouse at site of injection of commercial cholesterol in lard (15 per cent). 11th month. Experiment 10*d*. This tumour is now in its 63rd generation. × 55.

FIG. 2.—Same tumour as Fig. 1. × 290.

FIG. 3.—Sarcoma produced by commercial cholesterol in a C57 ♂ mouse. 20th month. Experiment 10*d*. × 55.

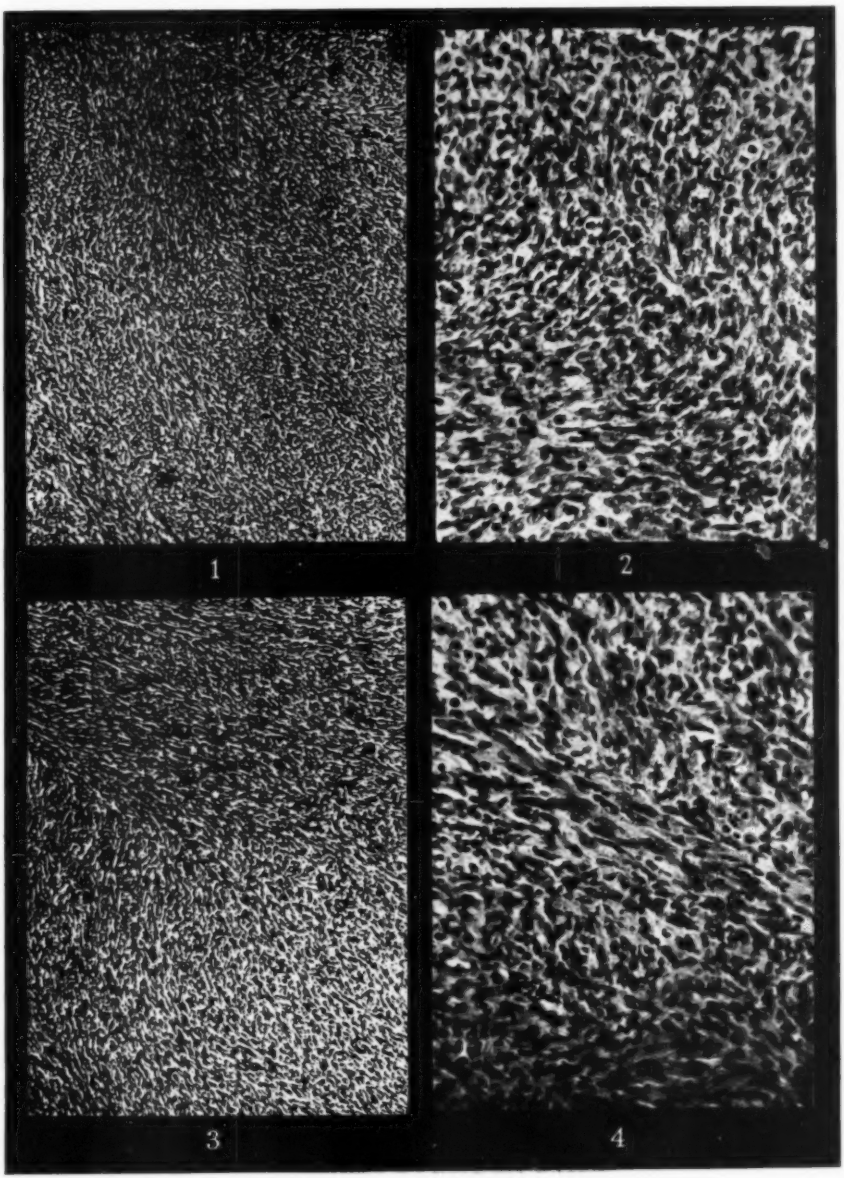
FIG. 4.—Same tumour as Fig. 3. × 290.

FIG. 5.—Sarcoma produced by commercial cholesterol in a MRC ♂ mouse. 23rd month. Experiment 10*d*. × 55.

FIG. 6.—Sarcoma produced by cod liver oil in a C57 ♀ mouse. 17th month. Experiment 10*b*. × 55.

FIG. 7.—Sarcoma produced by a fraction from lung-kidney-muscle of non-cancerous human subjects. The fraction had been saponified 3 times, crystallized from MeOH 3 times and once from acetone. 16th month. Stock ♂ mouse. Experiment 3*f*. × 55.

FIG. 8.—Sarcoma produced by lard in an old C57 ♀ mouse which was 16 months at the beginning of the experiment. 6th month. × 55.



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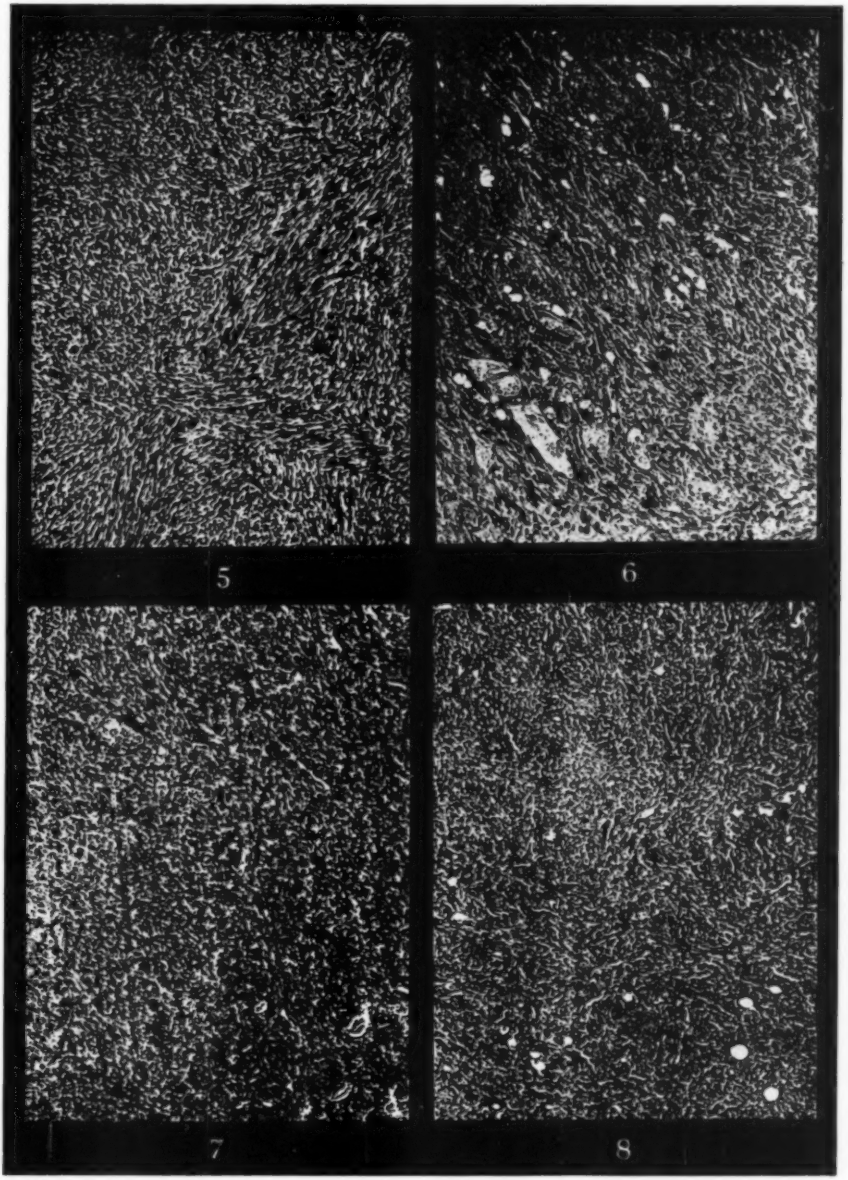

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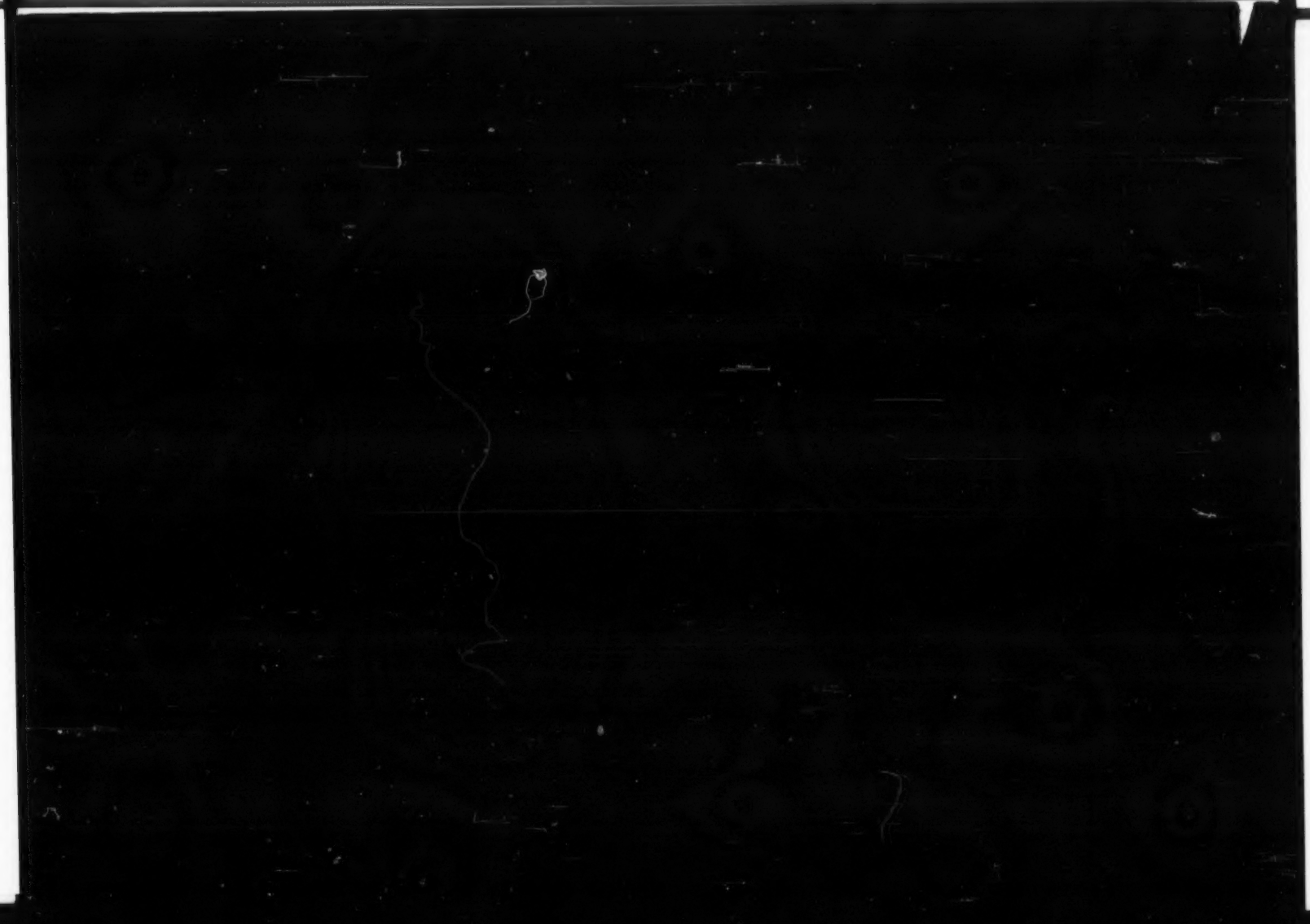


either absent in the reagents or are present only occasionally. If the carcinogen were derived from the spirit a constant carcinogenic factor would be present. An alternative explanation would be that, in the experiments just mentioned, the mice happened to be non-sensitive. The question of sensitivity will be discussed more fully later on.

3. *Fractionation of cholesterol rich* (c. 85 *per cent*) *carcinogenic fraction from human tissues.*

Since the carcinogen was concentrated in the *c.* 85 per cent cholesterol fraction of the unsaponifiable part of tissue, a number of sub-fractions were prepared and tested on mice, thus:

(*a*) Eighty-five per cent cholesterol fraction (fraction A) from pooled cancer livers was recrystallized from hot MeOH and cooled to room temperature instead of to — 10°; crystals and mother liquors were tested separately on 20 and 15 C57 mice respectively. In the 20th month a single tumour appeared in the "crystals" series (Table I).

Note.—In this paper "*cancer liver*" indicates the liver of a human subject who has died of cancer of *any* part of the body. "*Non-cancer liver*" indicates the liver of a human subject who has died of any cause *other* than cancer.

(*b*) Fraction A from mixed cancer livers, partly the same as those used for the preparation in (*a*), was crystallized from 80 per cent aqueous alcohol after adsorption from petrol ether on to a large column of Al_2O_3 eluted with petrol ether-benzene-MeOH (80–20–20). The 80 per cent aqueous alcohol crystals (glistening plates m.p. 130–135°) were tested on 10 C57 mice; the mother liquors sufficed for 5 mice. In the 19th month a sarcoma appeared in the crystal series.

(*c*) A more detailed fractionation of fraction A from mixed cancer livers was carried out as in Flow Sheet I.

The fractions A, B, C, D, E gave no tumours, although of 40 mice initially, 24 survived 21 months; for survival rate see Table I. The possible reasons for the absence of tumours are discussed below.

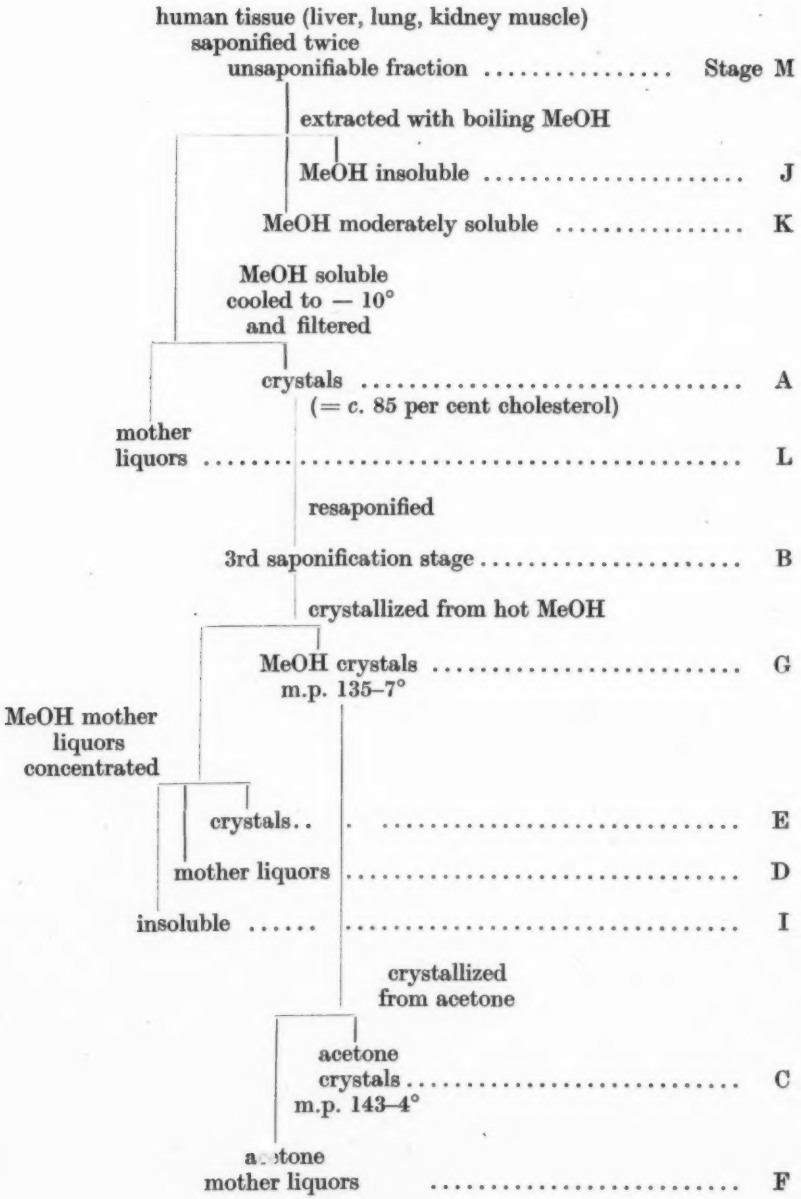
(*d*) A similar fractionation was made on another batch of cancer livers, and mice were injected with material from G, C, F, D, E and H. No tumours arose, although of 50 mice, 48 survived for 12 months, and 32 for 18 months. The test on F was repeated on a further 10 mice in which one tumour arose after 2 years.

(*f*) The — 10° fraction from human non-cancer lung-kidney-muscle (fraction A) had given 3 tumours in 25 mice, and was considered a potent carcinogen-containing fraction. It had been in the cold room for over 3 years and was then fractionated according to a simplified scheme (Flow Sheet 2).

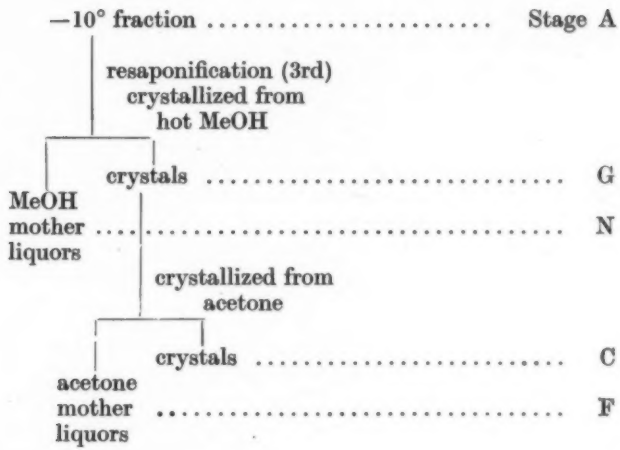
Of 52 mice at the beginning of the tests, 43 survived 12 months and 26, 18 months. Four sarcomas appeared, 3 in fraction G and 1 in C.

(*g*) The crude unsaponifiable fraction (M) of mixed livers which had been used in experiment (*d*) was fractionated and tested at J, K, L, G, A, C, F, M, I, using in all 110 mice, which lived well; 92 survived 12 months and 40, 18 months. Two tumours were produced, one by the fraction

FLOW SHEET I.


human tissue (liver, lung, kidney muscle)
saponified twice
unsaponifiable fraction ..... Stage M
extracted with boiling MeOH
MeOH insoluble ..... J
MeOH moderately soluble ..... K
MeOH soluble
cooled to — 10°
and filtered
crystals ..... A
(= c. 85 per cent cholesterol)
mother
liquors L
resaponified
3rd saponification stage ..... B
crystallized from hot MeOH
MeOH crystals ..... G
m.p. 135–7°
MeOH mother
liquors
concentrated
crystals.. ..... E
mother liquors ..... D
insoluble I
crystallized
from acetone
acetone
crystals C
m.p. 143–4°
acetone
mother liquors ..... F

FLOW SHEET 2.



obtained at the same level as in (*d*) (i.e. mother liquors of acetone crystals, (F)) and one by the crystals from which these mother liquors had separated (G).

(*h*) *Fractionation of 85 per cent cholesterol fraction via acetylation (in collaboration with Dr. C. W. Shoppee).*—In two earlier papers (Hieger, 1940, 1946) it was reported that the unsaponifiable fraction of the livers of Bantu natives contained carcinogen. A large batch of the crude unsaponifiable matter was worked up to stage A ; a part was kept for injection, and another part was acetylated and crystallized by careful seeding. The crystalline acetate was hydrolysed and the regenerated cholesterol tested. 20 mice were employed in each test. In the 12th month one sarcoma appeared in the A series, and in the 19th month one sarcoma developed in the cholesterol group.

(*i*) *Cholesterol-rich fraction from cream (cow's milk).*—Kennaway (unpublished communication) had found that the unsaponifiable part of cow's milk produced a sarcoma by injection into C_3H mice. A more concentrated source of the unsaponifiable fraction was obviously milk fat. 3 kg. cream was therefore saponified twice, extracted and crystallized from MeOH to give stage A fraction which was injected into 20 mice. Stage L fraction, i.e. mother liquors of A, were also injected into 10 mice. Two tumours appeared in the crystalline A fraction series in the 13th and 19th months ; no tumours were found at the site of injection in the L series.

(*j*) *Bile and gall bladder as sources of carcinogen.*—According to one hypothesis of human cancer, carcinogens are formed as a result of faulty steroid metabolism, consequently bile, which is a rich source of steroids, might well contain the aberrant factor.

Forty-five gall bladders from cancerous subjects were used; the bile was not separated, and some of the bladders were almost empty. Bladders and bile were saponified and worked up as before and the fractions tested at C, D, E, F. The experimental mice lived well; after 29 months a mouse of the F series (Flow Sheet I) developed a sarcoma at the site of injection.

(*k*) Liver from a lymphosarcomatous human subject gave a highly carcinogenic unsaponifiable fraction in some of the earlier experiments (Hieger, 1940), and the tissues of humans with this type of cancer may possibly be rich sources of carcinogen Three livers from lymphosarcoma cases were saponified, fractionated into stages A, J, M, L, and injected into 6, 5, 11 and 6 mice of crossed C3H × C57 strain, which survived well, but no tumours developed.

Experiment 4.

The 85 per cent cholesterol fraction (A) of cancer livers which had given 2 sarcomas in 10 stock mice was stored in the cold room for $2\frac{1}{4}$ years, then tested on 10 C57 mice. No tumours arose, although 3 mice survived 24 months.

Experiment 5.

Dr. Mead, of British Drug Houses Ltd., kindly sent us for testing 5 fractions consisting chiefly of cholesterol obtained from cattle liver in the preparation of vitamin A concentrates. 119 mice were used in all for these tests but they did not live well; 60 survived for 12 months and 18 for 18 months; no tumours resulted.

Experiment 6.—*To test if commercial cholesterol could replace the "cholesterol-rich (c. 85 per cent) fraction."*

Mixed human livers, some cancer, some non-cancer, were saponified and worked up to give the −10° (i.e. 85 per cent cholesterol) fraction (A) and also the mother liquors of this fraction (L). Commercial cholesterol was then added to L to give the same concentration of sterol as constituted the unsaponifiable fraction before the separation of the crystalline part A. Three series of mice were set up:

(1) injected with −10° fraction (A);
(2) ,, ,, mother liquors of −10°, i.e. L;
(3) ,, ,, L to which the appropriate amount of cholesterol had been added.

Of 32 mice originally 28 survived 12 months, and 14, 18 months; no tumours resulted.

7. *Effect of solvent.*

Sesame oil is commonly used as a solvent for the injection of compounds undergoing tests for carcinogenic activity. Gardner (unpublished communication) reported that he found a single sarcoma in control mice injected with this solvent. Steiner, Warren Stanger and Bolyard (1947) obtained one sarcoma in

145 mice injected with tricaprylin, and Harris (1941) found that wheat germ oil gave rise to sarcoma on injection into rats and mice.

Two series of mice were set up ; one was injected with sesame oil containing 10 per cent commercial cholesterol, and one with the oil alone as control. One sarcoma appeared in the first series in the 14th month in a C57 female, and one sarcoma of fibrous type in the second in the 22nd month in a stock female (Experiment 7, Table I).

When over 300 control tests had been carried out on lard alone without the production of a single tumour, a sarcoma arose at the site of injection in a C57 mouse first injected with lard when already 16 months old. The latent period was 6 months, the shortest observed here in all the tests on carcinogens of biological origin.

Old mice were used in this experiment to see whether the prolonged latent period for tissue carcinogens was equivalent to the interval required for some essential preliminary change in the tissue which prepares it for carcinogenesis, and whether the natural ageing of tissue could in some degree be a substitute for the latent interval.

Earlier experiments in this Institute had shown that sarcoma may occur in rats injected with lard alone when strict precautions were taken against contamination by hydrocarbons (Burrows, Hieger and Kennaway, 1932, 1936).

8. *Fatty extract of Walker rat tumour.*

Aptekman, King and Lewis (1943) induced sarcoma in rats by injection of the fatty fraction of Walker rat tumours. Their results were confirmed here by tests where the tumours were dried by storing in distilled spirit and extracting with benzene. The crude benzene extract was evaporated, and the residue without added solvent (lard) injected subcutaneously into 10 wistar rats. A parallel group of 10 rats were injected subcutaneously with lard alone. After two years one rat in each series developed a fibro-sarcoma, which grew with difficulty on transplantation. The tumour in the lard series was more malignant than the tumour in the Walker extract series. Both tumours arose in the subcutaneous tissue at the same level as the injection site, but on the opposite side of the body. This effect has been observed several times in experiments where the injected substance is very fluid, and is probably pressed round the subcutaneous spaces by the movements of the animal.

9. *Miscellaneous tissues.*

The ether extract of minced mammae from lactating mice (DBA of high mammary cancer strain) proved carcinogenic, for it gave 1 sarcoma in 10 mice (Hieger, 1941). An ether extract of the lactating mamma of the rabbit was evaporated and the oily material injected into 20 mice, but no tumours resulted. Two sarcomas were obtained by injection of an ether extract of human cancer liver (Hieger, 1946).

10. *Carcinogenic activity of commercial cholesterol.*

The carcinogenic activity of commercial cholesterol (about 95–98 per cent pure sterol) has been briefly described (Hieger, 1947). Tests of the commercial product dissolved in lard were begun when it was found that the fractions of

TABLE I.—*Sarcoma Production by Injection of Lipoid Substances.*

Experiment.	Substance.		Mouse strain.	Mice at start.	Survivors (months). 12.	15.	18.	21.	24.	30.	Total sarcomas.
3*a*	Livers from cancerous subjects. Fraction A: crystallized from MeOH → Crystals		C57	20	14	13	10	*9	5	1	1
	→ Mother liquors		„	15	14	11	10	8	0	0	0
3*b*	Livers from cancerous subjects. Fraction A: passed through Al_2O_3 column, crystallized from 80% alcohol → Crystals		C57	10	8	8	8	*6	5	0	1
	→ Mother liquors		„	5	4	4	4	4	4	0	0
3*c*	Livers from cancerous subjects.	Fraction A	C57	9	8	6	5	2	1	0	0
		„ B	„	11	10	10	10	10	9	0	0
		„ C	„	10	10	10	10	5	5	0	0
		„ D	„	5	5	5	4	4	3	0	0
		„ I	„	5	4	4	3	3	0	..	0
		(See Flow Sheet 1)									
3*d*	Livers from cancerous subjects.	Fraction G	C57	10	9	9	8	7	1	0	0
		„ C	„	10	10	10	10	8	6	0	0
		„ F	„	20	17	16	8	7*	3	0	1
		„ E	„	10	10	10	8	5	4	0	0
		„ D	„	5	5	5	5	0	..	..	0
		„ I	„	5	5	5	0	..	..	..	0
		(See Flow Sheet)									
3*f*	Lung-kidney-muscle from non-cancerous subjects.	Fraction A	Stock	10	9	4	3	2	0	..	0
		„ G	„	10	9	8	5	4*	*0*	..	3
		„ D	„	10	∂	8	6	2	0	..	0
		„ C	„	12	8	7*	5	3	0	..	1
		„ F	„	10	8	8	7	3	0	..	0
3*g*	Livers from cancerous and non-cancerous subjects.	Fraction M	Stock	15	10	9	4	1	1	0	..
		„ A	„	15	12	9	5	0	..	..	..
		„ K	„	10	8	7	3	0	..	..	..
		„ J	„	15	12	7	5	0	..	..	..
		„ G	„	10	*8	7	5	1	0	..	1
		„ L	„	15	12	10	5	0	..	..	..
		„ D	„	10	10	9	6	2	0	..	..
		„ F	„	5	4	3*	2	0	..	..	1
		„ C	„	15	14	10	5	2	1	0	..
3*h*	Livers from Bantu natives.	Fraction A									
	Fraction A acetylated, cholesteryl acetate crystallized out and cholesterol regenerated . .		C57	20	8*	5	2	2	..	..	1
			„	20	7	7	1*	1	..	..	1
3*i*	Cream (cow's milk).	Fraction A	C57	20	13*	11	11*	10	4	0	2
		„ L	„	10	9	9	4	0	..	..	0

TABLE I—(*cont.*).

TABLE I—(*cont.*).

Experiment.	Substance.		Mouse strain.	Mice at start.	Survivors (months). 12.	15.	18.	21.	24.	30.	Total sarcomas.
3*j*	Bile and gall bladders.	Fraction L	C57	10	10	8	4	3	3	0	0
		„ K	„	10	10	8	8	6	5	0	0
		„ C	„	10	10	10	8	3	2	2	0
		„ F	„	10	8	8	7	6	5	*3	1
3*k*	Livers from lymphosarcoma cases.	Fraction M	Hybrid	6	5	..	..	..	4	3	0
		„ J	„	5	5	..	..	..	3	2	0
		„ L	„	6	3	..	..	..	2	1	0
		„ A	„	11	9	..	..	..	7	2	0
6	Mixed livers (cancer + non-cancer). Substitution of A (i.e. 85% cholesterol fraction) by commercial cholesterol (C.C.).	Fraction A	C57	10	10	9	3	2	2	0	0
		„ L	„	10	9	8	5	4	0	..	0
		L + C.C.	„	12	9	8	6	4	2	0	0
		C.C. *S.O.*									
7	Commercial cholesterol (C.C.) + Sesame oil (S.O.).	10% 90%	C57	10	16	*15	2	1	—	..	1
			Stock	10							
		0 100%	C57	10	13	12	2	1*	—	..	1
			Stock	10							
10*a*	Commercial cholesterol refluxed with KOH and (*a*) alcohol, or (*b*) spirit.	*a*	C57	10	9	3	2	1	1	0	0
			Stock	10	7	0	..	..	..	..	0
		b	C57	10	10	10	10	7	2	0	0
			Stock	10	9*	*0	..	..	..	..	2††††
10*b*	Commercial cholesterol (C.C.) + Cod liver oil (C.L.O.), or linseed oil (L.O.). Final mixtures made up as 15% solutions in lard.	*C.C.* *C.L.O.* *L.O.*									
	I	1 g. 12 "drops" 0	C57	10	10	10	9	*6*	5*	..	3
	II	1 g. 2 „ 0	„	10	10	9	9	8	8	..	0†
	III	1 g. 0 12 drops	„	10	10	10	10	8	4	..	0
	IV	1 g. 0 2 „	„	10	10	9	7	6	3	..	0
	V	0 1·0 c.c. 1·0 c.c.	„	10	9	4	2	1	..	..	0
	VI	0·5 g. 1·0 „ 1·0 „	„	10	10	10	8	6	6	..	0
	VII	6 g. 0 0	C57	15	20	*14	14	..	..	..	1 (Stock ♂)
			Stock	15							
	VIII	6 g. 1·2 c.c. 0	C57	10	13	*10*	10	*4	..	..	1††††
			Stock	15							3 (Stock ♂, ♂, ♂)
	IX	1·2 g. 6 „ 0	C57	10	*8	8	7	..	..	..	1††
			Stock	15							(C57 ♂)
	X	0 6 „ 0	C57	10	12	12*	9	..	..	..	1†
			Stock	15							(C57 ♀)

TABLE I—(*cont.*).

Experiment.	Substance.	Mouse strain.	Mice at start.	Survivors (months). 12.	15.	18.	21.	24.	30.	Total sarcomas.
10c	Commercial cholesterol crystallized from acetone — Least soluble fraction	C57	10	13*	11	**11	..	..	..	3 (Stock ♂, ♀ and C57 ♀)
		Stock	10							
	Mod. soluble	C57	11	15*	12	9	..	..	..	1 (Stock ♂)
		Stock	10							
	Mother liquors	C57	12	13	12	10	..	..	..	..
		Stock	10							
10d	Commercial cholesterol§	C57	5	*4	1	0	..	..	..	1
		,,	5	0	..	..	..	..	..	0
		,,	6	3	3	3	3	3*	0	1
		,,	6	4	4	0	..	..	..	0
		,,	6	0	..	..	..	..	..	0
		CBA	5	3	2	0	..	..	..	0
		,,	5	5	2	1	0	..	..	0
		C57 ♀	5	5*	4	4	1	0	..	1
		,, ♀	5	5	5	2*	1*	..	..	2
		C3H	5	2	1	0	..	..	..	0
		,,	5	2	0	..	..	..	..	0
		MRC ♀	6	3	3	3	0	..	..	0
		,, ♂	5	4	3	2	2*	0	..	1
		,, ♂	5	5	4	3	2*	2	0	1
		,, ♂	5	4	0	..	..	..	..	..
		C57 ♂	5	4	4	..	..	..	..	..
		Stock ♂	5	0	..	..	..	..	..	..
		,, ♂	5	3	0	..	..	..	..	..
		,, ♀	5	4	3	..	..	..	..	..
		,, ♀	4	4	3*	2*	..	..	..	2
		,, ♂	5	4	3*	..	..	..	..	1

* Each asterisk represents one sarcoma. The position of the asterisk on the time-grid indicates the latent period.

† Indicates histological changes at the site of injection suggestive of incipient sarcoma production. Thus in bottom line of Experiment 10*a* (10 Stock mice) sarcomas arose in 2 mice and suggestive changes were observed in 4 others.

In Experiment 10*b*, V the mixture of oils was first irradiated with U.V. light before injection.

§ For a more complete statement of the results with cholesterol see Table II.

tissues which were richest in cholesterol were also the most potent carcinogenically. Up to the present 25 sarcomas have been obtained in 436 mice by commercial cholesterol or some fraction of it, in solution in lard or in other solvents (Table I (10*d*), and Table II).

TABLE II.—*Sarcoma Production by Commercial Cholesterol.*

	Material.*	Number of mice.		Sarcomas.
		Initial.	12 months.	
1.	Commercial cholesterol	213	77	11
2.	,, ,, in sesame oil	20	16	1
3.	,, ,, + cod liver oil	100	71	7
4.	,, ,, (after refluxing with KOH in spirit (or alcohol))	40	35	2
5.	,, ,, crystallized from acetone . . .	63	41	4
	Total	436	240	25

* Injections of 15 per cent solutions in lard except for No. 2.

10*a*. *Effect on cholesterol of refluxing with KOH and alcohol.*

The possibility that carcinogens are formed from cholesterol during saponification was tested as follows: 10 g. of commercial cholesterol (i.e. about the amount present in a human liver) was refluxed for 2½ hours with 400 g. KOH, 1 l. distilled alcohol or spirit and enough water to imitate the conditions in the saponification of a liver; the mixture was extracted with ether and injected into mice as before. A preliminary statement (Hieger, 1947) was made, but further series of mice have been used (10*a* in Table I).

Four groups of mice were set up:

a. 10 C57 mice injected with cholesterol saponified in distilled spirit.
b. 10 stock ,, ,, ,, ,, ,,
c. 10 C57 ,, ,, ,, ,, alcohol.
d. 10 stock ,, ,, ,, ,, ,,

In the 14th month in group *b* 2 mice developed sarcomas, and pre-neoplastic changes were seen at the site of injection in 4 other mice.

Thus in group *b*, 6 of 10 mice had either developed sarcoma or might have done so if they had lived longer. The two sarcomatous mice and two of the incipiently sarcomatous mice were from the same box of 5 mice.

One cannot say why only group *b* responded so readily, but (1) in a fair number of experiments the saponification of tissue has been done in spirit but the products have produced no tumours, showing that spirit is not essential for carcinogenesis by the unsaponifiable product; (2) the absorption spectra of the two final products (cholesterol saponified in spirit and in alcohol) are closely similar; and (3) while the substance was strongly carcinogenic in group *b* (stock mice), the same preparation was inactive in group *a* (C57). Consequently it must be supposed that the *b* group of mice were either—

(1) Peculiar genetically; (2) peculiar congenitally; (3) had become infected or (4) had become contaminated with carcinogen.

(4) is, on the whole, not very probable, because:

The 1946 paper reported that in tests on 18 subfractions of the unsaponifiable fraction of human tissue, partly from cancerous, partly from non-cancerous

subjects, of the total yield of 8 sarcomas, 7 were produced by fraction A made from 3 different sources and 1 from an M fraction (Flow Sheet 1). The concentration of 7/8ths of the sarcomas in one particular fraction strongly suggests that the fraction itself was responsible and not a chance contamination, for it would be highly improbable that contamination would occur only in the boxes, holding 5 mice each, containing those treated with the A fraction.

The data in Table III are the results when a fraction was divided into 3 or more sub-fractions. The concentration of tumour production in a few fractions, particularly F, G and A, supports the conclusion in the foregoing paragraph, namely, that contamination will not explain satisfactorily the incidence of sarcomas. Contamination only in those boxes where these particular fractions were used would be very unlikely, since each box contains 5 mice initially and the experiments on these fractions were started over a period of 4 years, during which time the boxes were changed many times (Table III).

TABLE III.—*Showing Trend of Concentration of Carcinogenic Factor During Fractionation.*

Fraction (see Flow Sheet 1).	Number of samples derived from different batches of tissue.	Number of mice at start.	Survivors at 12 months.	Number of sarcomas.		Number of samples which gave positive results.
					%	
A	11	175	110	10	6	5
M	4	52	41	1	2	1
J	4	40	25	0	0	0
L	8	141	101	0	0	0
G	3	30	26	4	13	2
C	5	57	52	1	2	1
F	4	45	37	3	7	3
I	2	10	9	0	0	0
D	3	25	24	0	0	0
E	1	10	10	0	0	0
K	2	20	18	0	0	0

10*b*. *Effect of addition of oils on carcinogenic potency of commercial cholesterol.*

The activity of commercial cholesterol suggested several possibilities; for example:

(*a*) That cholesterol is indeed a feeble carcinogen.

(*b*) That commercial cholesterol as well as the cholesterol-rich fraction obtained from a number of sources contains a small amount of a potent carcinogen.

(*c*) That a combination of cholesterol, as carrier, and an active agent are essential for carcinogenesis.

(*d*) That all the preparations of cholesterol used are contaminated with atmospheric carcinogen (i.e. benzpyrene from soot).

(*e*) That carcinogens of biological origin such as lipids and particularly preparations rich in cholesterol evoke neoplasia, but only in the most susceptible members of groups of experimental animals.

Tests of hypotheses (*a*) and (*b*) are to be described more fully in a forthcoming paper. Highly purified cholesterol, prepared by Dr. C. W. Shoppee, using bromination, crystallization of acetate and chromatography, has been

injected into 25 mice; the experiment had been in progress for 22 months without result as yet. (*b*) is being tested by concentrating the impuritities in commercial cholesterol 3 times and in a second experiment 10 times, and injecting the products. These experiments have been in progress for 11 months without result as yet. (*c*) was investigated by mixing oils containing reactive double bonds (i.e. unsaturated) with commercial cholesterol to see if the activity could thereby be increased (Table I, experiment 10*b*).

Cod liver oil certainly increases the potency of cholesterol dissolved in lard, but since at least one sarcoma has now been obtained with cod liver oil alone (Table I, 10*b*, series x), it is uncertain whether the oil is a co-carcinogen or a carcinogen.

10*c*. *Effect of simple crystallization on the carcinogenic potency of commercial cholesterol.*

The batch of cholesterol used in the preceding experiment was crystallized from acetone to give 3 fractions:

(1) Least soluble.
(2) Moderately soluble.
(3) Mother liquors, which is obviously different from (1) and (2), for (3) is softer, yellower, more soluble in lard and smells of candle wax.

The three fractions were injected as usual into 20, 21 and 22 mice respectively; 3 sarcomas appeared in the fraction 1 (least soluble) series and 1 in fraction 2 series in the 14th, 18th, 18th and 15th months.

10*d*. *Sarcoma production by commercial cholesterol.*

Table II shows the total of sarcomas produced by commercial cholesterol.

10*e*. *Compounds related to cholesterol.*

Compounds related to cholesterol and particularly those which might be present in samples of the impure sterol were also tested, namely, isocholesterol, isolumisterol, cholesterylene (2 different preparations and also the mixture of compounds present in the mother liquors after crystallizing out the cholesterylene), ergosterol, cholestene, 7-dehydrocholesterol (2 different preparations, one a commercial product containing 60 per cent of the pure dehydrocholesterol), a mixture of epi- and allo-cholesterol, vitamin D_3, and 7-ketocholesterol. Except for those treated with 7-ketocholesterol all the mice have now died without tumours being produced; 90 survived 12 months out of 100 initially, and 56 survived 18 months. The test on 7-ketocholesterol has now been in progress for 17 months, without result.

11. *Pre-neoplastic changes at the site of injection of lipid.*

Since the detection of only a few tumours could be important in deciding the technique of further fractionation, it was essential not to miss any tumours not visible macroscopically. The remaining mice (now 700) are examined post mortem by sections through the fat vesicle, so that any pre-neoplastic changes might be detected. In 15 of the 700, changes were seen suggestive of pre-sarcomatous change at the site of injection.

12. *Steiner's investigations.*

In a recent elaborate report Steiner, Warren Stanger and Bolyard (1947) give further data on sarcoma production by unsaponifiable fractions in three strains of mice, Albino, A, and C57 (Table IV, A):

TABLE IV, A.—*Experiments of Steiner, Warren Stanger and Bolyard* (1947):—*Sarcoma Production by Unsaponifiable Fraction of Tissues.*

Experiment.	Unsaponifiable fraction of—	Number of cases (pooled).	Mice at start.	Mice at 12 months.	Sarcomas.
	Cancer cases:				
1	Human kidney	61	50	39	0
2	,, spleen	54	50	47	5
3	,, colon	54	49	45	0
4	,, heart	63	43	19	1
5	,, liver	18	101	45	17
	Non-Cancer cases:				
6	Human kidney	58	50	44	1
7	,, spleen	51	50	40	0
8	,, colon	51	47	45	0
9	,, heart	35	31	7	0
10	,, liver	15	120	39	44
11	Human liver (stillborn infants)	40	39	34	3
12	Pig liver	—	115	82	4
13	Beef liver	—	115	69	0
14	Pig heart	—	30	9	0
15	Tricaprylin controls	—	154	116	1
		Total	1044	680	76

The yield of local sarcomas at the site of injection (7·3 per cent) is of the same order as that obtained in this Institute, namely 4·7 per cent (63 sarcomas in 1298 mice, of which 936 lived 12 months). These 1298 mice are all those injected with unsaponifiable material of animal origin, including commercial cholesterol.

In earlier papers, Steiner (1942, 1943) gives results which, taken in conjunction with the data from his recent paper, can be expressed thus (Table IV, B):

TABLE IV, B.—*Experiments of Steiner, Warren Stanger and Bolyard* (1947):—*Sarcoma Production: Effect of Pooling of Unsaponifiable Fractions.*

Material.	Ratio: Sarcoma mice/total mice used.	
1. 8 cancer livers pooled	12/56 = 21%	mean 21%
2. 7 non-cancer livers pooled	5/63 = 8%	
3. 15 ,, ,, ,,	44/120 = 37%	
4. 18 cancer livers pooled	17/101 = 17%	
5. 37 ,, ,, tested separately (8 in 37 were active)	12/456 = 2·4%	
6. 30 non-cancer livers tested separately (6 in 30 were active)	10/440 = 2·3%	

Thus the effect of testing a number of livers (67) separately was to reduce the sarcoma production by a factor of 9, i.e. 9 times less than the potency of 48 livers pooled, which is of the order of expectation if it be noted that Steiner (1942) found 1 in 5 of his livers (cancerous and non-cancerous) to contain the active factor, and if it be assumed that pooling the livers had the effect of distributing the agent from the active livers among the inactive ones. Furthermore, it may be noted that from Steiner's (1942) experiments 1 and 2 one would conclude that

cancer livers are of the order of 2½ times as potent as non-cancer livers, but from experiments 3 and 4 it would appear that the ratio of potencies is reversed, leading to an overall discrepancy as high as 5 to 1. The writer would point out that three experiments (Nos. 10*a* and 3*f*, Table I and Experiment 4) carried out here suggest that unexplained differences in the susceptibility of the mice to biological carcinogens can completely alter conclusions as to the carcinogenicity or otherwise of a substance undergoing test.

TABLE V.—*Latent Period* (Months) of Sarcomas Produced in Mice by Injections of Lipoid Substances.*

Latent period (months).	Hieger (1940). Number of tumours.	Hieger (1946). Number of tumours.	Recorded in the present paper. Number of tumours.	Total.
6	—	—	1	1
7	—	—	—	—
8	—	—	1	1
9	—	—	—	—
10	1	—	—	1
11	2	—	1	3
12	2	—	2	4
13	1	—	2	3
14	—	1	6	7
15	1	2	1	4
16	—	1	4	5
17	1	—	3	4
18	2	—	2	4
19	2	1	3	6
20	1	1	3	5
21	2	—	3	5
22	1	—	4	5
23	2	1	3	6
24	—	1	2	3
25	—	—	1	1
26	—	—	—	—
27	—	—	—	—
28	—	—	—	—
29	—	—	1	1
			Total	69

* "Latent period" is taken as the interval between the start of the experiment and the grafting of the induced tumour. The actual initiation time for the tumour is, of course, shorter. The interval between detection and grafting is seldom more than a few weeks. Once the primary tumour is well started it grows with great speed.

Note.—The figures in the last column (the overall latent period) show a periodicity remarkable in such data. It is possible that at the large number of mice involved, nearly 2000, statistical laws begin to operate.

DISCUSSION AND CONCLUSIONS.

The experimental data given here and in earlier papers from this Institute, based on 69 sarcomas produced in a total of 1966 animals, present a number of difficult problems.

No completely satisfactory explanation of the following experimental findings is as yet available :

(1) Cod liver oil, lard and sesame oil produced at least 1 sarcoma each in 25, 350 and 20 mice respectively. Other work has shown that some fats can bring about sarcoma development in sensitive mice or rats.

(2) Commercial cholesterol in lard gave sarcomas in about 5 per cent of 213 mice (Table II).

(3) The mixture formed when cholesterol is refluxed with KOH and distilled spirit gave no tumours when injected into 10 C57 mice, but in 10 stock mice there arose 2 sarcomas, and in 4 other mice of the same series changes were detected, post-mortem, suggestive of pre-neoplasia, at the site of injection. A similar preparation where cholesterol was refluxed with KOH and alcohol gave no tumours when injected into 10 stock and into 10 C57 mice.

(4) Fractionation of an active fraction from tissues by simple crystallization does not always lead to any concentration of the active factor.

(5) Although several experiments showed that the addition of cod liver oil to commercial cholesterol increased its potency, yet in another experiment the crystallization of the commercial sterol from acetone, which removed some waxy constituents, *also* increased the potency.

The writer has attempted to reconcile these apparently contradictory findings by putting forward the following suggestions : Firstly, that the operation of little understood factors covered by the expression " individual variations of susceptibility of the experimental animals " is very important in determining the carcinogenic potency of the lipids, and secondly, that lipids and particularly sterols or closely associated compounds are slow-acting carcinogens. It is clear that large series of mice are required in this type of experiment where the agent is of low potency, and where, therefore, the presence or absence of especially sensitive individuals may well be the principal factor in the assessment of the potency of a test preparation, since in small series of mice the sensitive members may die first, leaving those which are comparatively insensitive.

The possibility is not quite excluded that lipids are often contaminated by traces of benzpyrene from air-borne soot.

Thirdly, the existence of about 300 synthetic carcinogens (hydrocarbons, azo compounds, stilbenes, etc.) suggests that carcinogenic potentiality is probably more widely distributed among chemical compounds than was at first anticipated, and thus there is no good reason for excluding compounds of the lipid group.

Fourthly, some of the experiments described here suggest that the carcinogenicity of lipid substances may either be enhanced or be inhibited by other lipids.

Fifthly, should the thesis proposed here, namely, that lipids are slow-acting carcinogens, be conclusively proved, then, " spontaneous " cancer or at least some forms of the disease could be considered as " caused " by body lipids existing locally in some peculiar chemical or spatial or temporal conditions.

The production of sarcoma by commercial cholesterol obviously casts some doubt on the conclusions which have been drawn from all previous experimental work where cholesterol was involved, and where almost all investigations have taken for granted that cholesterol is non-carcinogenic. The weakly carcinogenic potency of a number of other lipid substances and the unexplained variations of sensitivity of the animals used for testing underline the need for much further study in this field. The experiments described here suggest the question, Why has it required 20 years, that is, since the discovery of the carcinogenic hydrocarbons in 1929, to show that commercial cholesterol is carcinogenic ? The answer is obvious ; investigators in chemical carcinogenesis have become accus-

tomed to the use of powerful, quick-acting carcinogens, and as a result tumour induction by slow-acting carcinogenic substances has been dismissed as "non-specific."

Control experiments have, in the past, not been done on an adequate scale, and moreover, commercial cholesterol, having been unconsciously equated with the cholesterol in the body, leads to the inference that cholesterol is not a carcinogen since it is present universally. It might be permitted to note, however, that inferences cannot always satisfactorily substitute experiments.

SUMMARY.

1. A total of 69 sarcomas has been obtained at the site of injection of lipoid substances in approximately 2000 mice of C57 and MRC strains and commercial mixed stock mice. The average latent period was about 18 months.

2. 63 tumours were produced by unsaponifiable fractions derived from the tissues of human subjects (cancerous and non-cancerous) and from cattle; of the remaining 6 tumours, 5 were produced by non-saponified fat from animal sources and 1 from a plant source.

3. Commercial cholesterol gave 25 sarcomas in 436 mice.

4. No evidence was obtained to suggest that the carcinogenic substances in the unsaponifiable fraction is confined to the tissues of human subjects who have died of cancer.

5. The carcinogenic factor has not yet been isolated in a chemically pure state. Fractionation by simple crystallization led in one experiment to an inexplicable loss of potency.

6. The widespread occurrence of a carcinogenic factor in unsaponifiable fractions from many biological sources suggests that it is either cholesterol itself, or a combination of cholesterol and a small proportion of a frequently occurring co-carcinogen.

7. In such experiments the possibility of contamination with benzpyrene from air-borne soot must be borne in mind.

8. The variations of susceptibility in the experimental mice constitutes a most important factor in determining the potency of carcinogens of biological origin.

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INVESTIGATIONS ON THE SERUM PROTEINS IN RATS DURING THE DEVELOPMENT OF HEPATIC TUMOURS DUE TO FEEDING p-DIMETHYLAMINOAZOBENZENE.

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THE findings on the concentrations of proteins in serum of tumour hosts differ widely. In experimental animals low total proteins have been reported. For human patients most investigators report low total proteins, but normal or increased serum proteins have also been often found (Stern and Willheim, 1943). It was considered that the excess protein might be derived from the disintegration of tumour tissue. Undoubtedly the actual concentration of the total serum proteins will depend on the site and the extent of the growth, secondary involvements, infections, state of nutrition, etc. Fractionation of the serum proteins by different methods showed deviations from the normal pattern, generally not specific or constant enough to be of diagnostic significance. Recently Seibert, Seibert, Atno and Campbell (1947) published the results of electrophoretic investigations on 23 sera from cancer patients. Generally the albumin was lowered, the α_2-globulin increased, the γ-globulin decreased, except in cases with liver involvements, in which the γ-globulin was increased.

It was decided to see if any correlation in the changes in the serum proteins existed with the development and size of tumours. Rats in which hepatic tumours were induced by feeding p-dimethylaminoazobenzene (pDA) were considered to be suitable for this investigation.

EXPERIMENTAL.

Twenty-six female and 16 male blackhooded rats were fed 0·06 per cent pDA in addition to a semi-synthetic diet consisting of 53 per cent starch, 17 per cent casein and 30 per cent fat (Ministry of Food cooking fat) of its caloric value. The diet was supplemented with cod liver oil, and greens once weekly, and 2–3 g. bread (80–85 per cent extraction) daily. Thirty-four female and 12 male control rats received the same diet without pDA or a mixed diet of rat cubes, bread, and once weekly greens. The diet and water were given *ad libitum*. All the rats grew at a normal rate. The rats receiving pDA were killed at intervals, starting in the 7th month of the experiment, when palpable tumours were present in some of the rats; the experiment was terminated in the 14th month of feeding pDA. The control animals were killed at different intervals when 6 to 12 months old. All rats were killed by dislocating the neck. Blood was withdrawn from the heart immediately, and the concentration of the serum protein was estimated by a modified Linderström-Lang gradient (Hoch and Marrack, 1945).

It has been repeatedly observed (Metcoff and Favour, 1944; Leathem, 1947; Lippman, 1948) that in young rats the serum protein concentration rises with

age, and is highest in the mature animal. The value for total proteins obtained in the present experiments, in which only mature animals were employed, are higher than those found by Lippman for mature animals, but lower than the values of Metcoff and Favour. The latter authors used the copper sulphate method. In the present experiment the equation $P = 361(S - 1{\cdot}007)$, derived by Hoch and Marrack for human sera, was used. The validity of this equation for rat sera was checked by micro-Kjeldahl estimations. The deviations of the values obtained by the specific gravity method were within + 6·5 and − 3·5 per cent in 10 cases.

The sera were fractionated by electrophoresis in the Tiselius apparatus with the cylindrical lens schlieren optical system (Thovert, 1914; Philpot, 1938; Svensson, 1939). The medium size 11 ml. cell was used. The buffer solution contained 0·0327 M Na_2HPO_4 and 0·00175 M NaH_2PO_4, and had a pH of 8·0 and ionic strength of 0·1. The serum was diluted with this buffer to a protein concentration of about 1·2 g./100 ml., with a few exceptions, and the solution was dialysed in the ice box for 1–3 days against 2 l. of buffer. The solutions were electrolyzed at 8·4 V./cm. for about 70 minutes, at which time the separation of the components was optimal for analysis. Both diagonal wire and edge were used for recording the patterns. The position of the centre line in the pattern given by the wire is independent of the degree of illumination, while underexposure, caused by pigments or turbidity, of parts of the pattern given by the edge, results in a distortion of the curve (Svensson, 1939). The patterns obtained with the edge, however, are better suited for reproduction. Before every run the base lines from both limbs were photographed. The final patterns and the base lines were traced under magnification, superposed in such a way that those parts which did not show any elevation of the base line coincided. Both the ascending and descending patterns were analysed. The areas were divided by the method of Pedersen (1940) as used by Longsworth (1946). The α- and the γ-globulins appear frequently as broad regions (Moore, 1945), which by overlapping with the β-globulin peak, made the separation of the globulins into individual groups uncertain. The uncertainty in the values given for the α-, β- or γ-globulins was ± 15 per cent or less. Wherever the uncertainty was greater, the combined areas of β- and γ-globulin were measured. The proportion of the total area made up by the albumin peak, into which the small component migrating faster than the albumins was included, could be read to within ± 2 or 3 per cent.

RESULTS.

The values for the total serum proteins of the rats with hepatic tumours are significantly higher than those of the control animals in both sexes (Table I).

TABLE I.—*Total Serum Proteins in Control Rats and in Rats Fed pDA.*

Sex.	Diet.	Tumour.	Number of rats.	Body weight, g.		Serum proteins, g. /100 ml.			
				Range.	Mean.	Range.	Mean.	σ.	P.
♂	Control	−	34	130–250	158	6·0–8·65	7·05	0·15	—
♂	„ +pDA	+	16*	137–225	175	7·2–9·5	7·99	0·17	<0·01
♂	„ +pDA	−	8	137–195	164	6·5–7·4	7·13	0·12	0·7
♀	„	−	12	150–254	193	6·6–8·1	7·18	0·11	—
♀	„ +pDA	+	10*	170–267	238	7·25–8·75	8·21	0·14	<0·01
♀	„ +pDA	−	5	185–260	230	6·6–7·6	6·83	0·31	0·3

* Animals killed when acutely ill are not included.

Fig. 1 and 2 show the distribution of the serum protein values in (A) rats fed pDA, which did not develop hepatic tumours; (B) rats with hepatic tumours of various sizes—from microscopical to large tumours (liver + tumour up to 50 g.); and (C) rats with tumours, obviously very ill when killed. It is evident that the

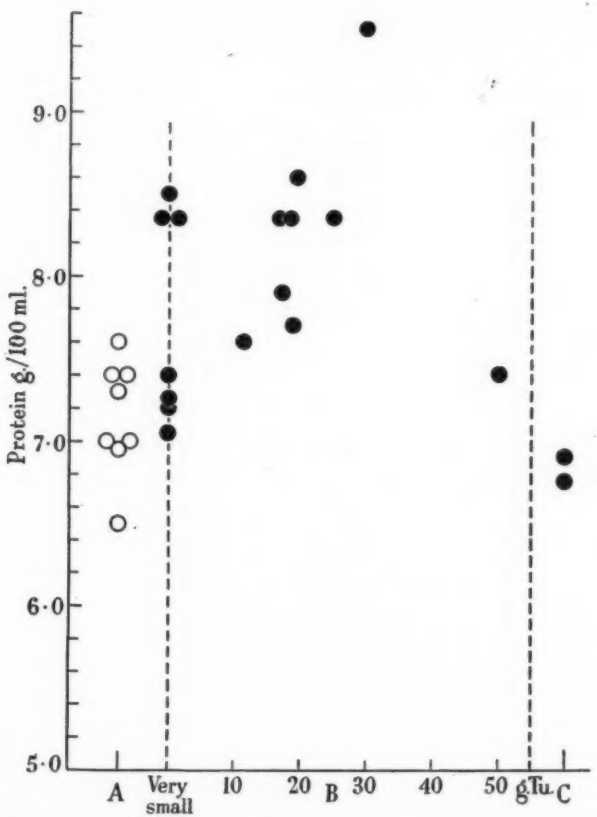

FIG. 1.—Females. Distribution of the serum protein levels in rats fed pDA. A = rats without hepatic tumours; B = rats with hepatic tumours. The values are arranged according to the size of the tumours. When the tumour was large and could not be separated the total weight of liver and tumour is given. C = rats with tumours killed when acutely ill.

proteins are already increased in many cases when the tumour is of microscopical dimensions, that the increase is independent of the size of the tumour, and that the serum protein drops when the rats are seriously ill. It is noteworthy that the well-being of the rats does not depend upon the size of the tumour and is not disturbed, even when metastases are present in the omentum or when the rat has a considerable ascites. Such animals are seen to feed comfortably. Only

when the lungs or intestines become involved do the animals become acutely ill. Ten to 30 ml. of ascites was found together with high serum protein.

The sera from 6 treated and 10 control animals were analysed by electrophoresis, and the results are given in Table II and Fig. 3. No difference in the patterns was found between the normal albino and blackhooded rats, on the semi-synthetic diet, or on the mixed laboratory diet. All these patterns showed a double albumin peak, the components of which were separated to varying extent.

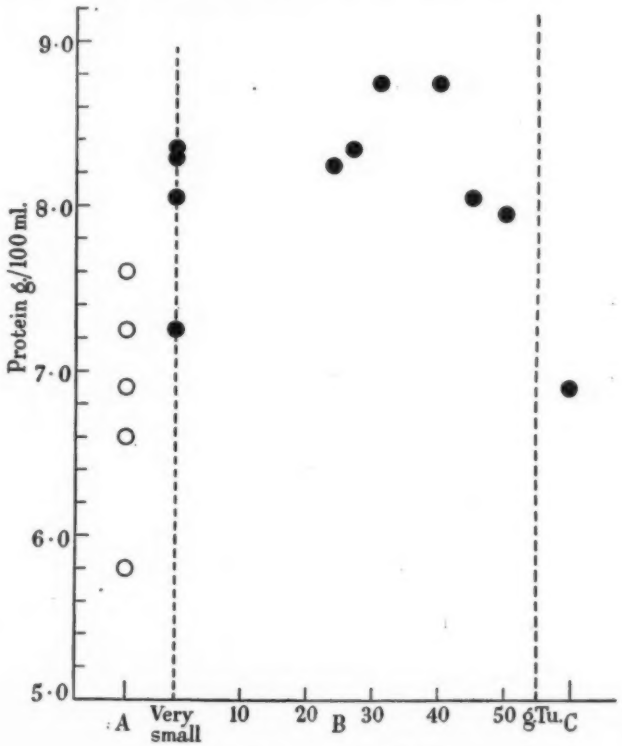

FIG. 2.—Males. Same as Fig. 1.

In the descending pattern the peak of the slower albumin component was in some cases higher than the corresponding peak in the ascending pattern, and in some cases this region in the descending side indicated gravitational instability, reminiscent of the β-anomaly, which is often seen with normal human sera. In contrast the patterns of sera from rats which developed hepatic tumours due to the ingestion of pDA showed only one single albumin peak. In No. 15 there was a small amount of a second albumin component. Rat 16, which had been fed pDA for the same length of time as the other animals and which did not have a tumour, gave a pattern in which the albumin showed two components. In No. 14 the

presence of a second albumin peak was doubtful. The gravitational disturbance on the descending side was seen in Cases 15 and 16, and to a smaller extent in Cases 13 and 14.

The concentration of the total globulins was significantly increased in the group fed pDA, while the concentration of the albumin remained unchanged. The increase occurred mainly in the β- and γ-globulins with the exception of Case 12. The increase was very marked, and the ranges for the treated and untreated groups hardly overlapped. The increase in the α-globulin was not significant. In a number of cases the α-globulin migrated with a single boundary; in others two well-separated peaks were seen in both the ascending and the descending patterns. In one case a single α-globulin peak broke up into two or

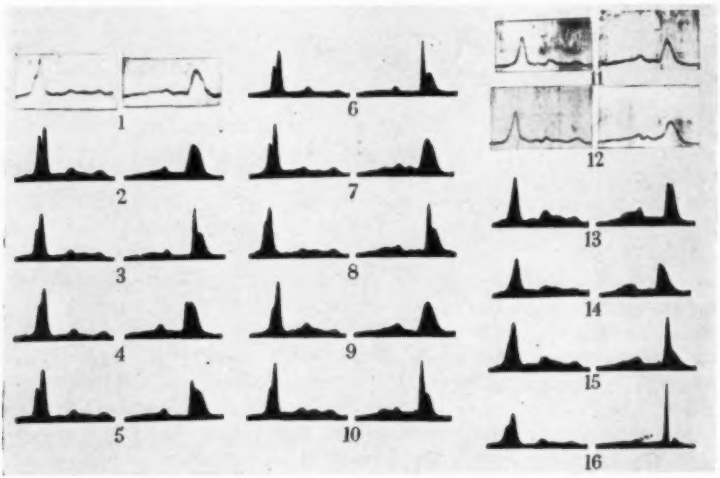

FIG. 3.—Electrophoretic patterns of rat sera. 1–10, control rats, 11–16, rats fed pDA. Left = ascending, right = descending patterns.

three peaks in the ascending limb, remaining single in the descending limb-and convectional disturbances were observed near the α-globulin region of the ascending limb, but not in the descending or any other part of the ascending limb. (Similar disturbances near the α_2-globulin region have been observed with some human sera in more concentrated solution in the same buffer.) These disturbances were not due to heat effects caused by the electric current, but probably to the formation of a gravitationally unstable region. The variation in the shapes of the normal patterns is remarkable. Of the 10 normal patterns, hardly two can be said to be similar in all details.

DISCUSSION.

The present experiment was concerned with the changes in the serum proteins in rats with hepatic tumours only, and advantage was taken of the possibility of obtaining samples of blood at the different stages of tumour development.

TABLE II.—*Electrophoretic Patterns of Rat Sera.*

Number.	Sex.	Total protein.	Albumin.*	Globulins.				Number of albumin components.	Conc. in elect. cell.
				Total.	*a.*	*β.*	*γ.*		
Controls:									
1	♂	7·15	4·7 (65·5)	2·5 (34·5)	0·7 (9·5	0·6 9·0	1·2 16·0)	2	0·75
2	♀	7·27	5·0 (68·0)	2·3 (32·0)	0·5 (7·0	0·8 10·9	1·0 14·2)	2	1·2
3	♀	8·14	5·4 (66·1)	2·7 (33·9)	0·7 (8·9	0·9 10·8	1·1 14·2)	2	1·2
4	♂	7·38	4·8 (65·3)	2·6 (34·7)	0·8 (10·4	0·9 12·1	0·9 12·2)	2	1·1
5	♂	7·62	5·0 (65·1)	2·6 (34·9)	0·7 (9·3	0·8 10·8	1·1 14·8)	2	1·2
6	♀	7·25	5·2 (71·0)	2·0 (29·0)	0·6 (8·8	0·7 10·0	0·7 10·2)	2	1·1
7	♂	7·04	4·5 (63·9)	2·5 (26·1)	0·7 (10·4	0·8 11·4	1·0 14·3)	2	1·2
8	♀	6·90	4·4 (63·7)	2·5 (36·3)	0·6 (9·0	1·9 27·3)		2	1·2
9	♀	7·45	4·7 (62·5)	2·8 (37·5)	0·6 (8·3	2·2 29·2)		2	1·2
10	♂	6·60	4·1 (62·1)	2·5 (37·9)	0·5 (8·2	0·9 13.0	1·1 16·7)	2	1·2
Rats fed pDA:									
11	♂	8·35	5·0 (59·2)	3·4 (40·8)	0·9 (10·7	1·3 15·1	1·2 15·0)	1	0·85
12	♀	7·40	4·2 (56·8)	3·2 (43·2)	1·3 (17·6	1·9 25·6)		1	1·2
13	♂	8·65	4·6 (53·7)	4·0 (46·3)	0·8 (9·7	1·2 13·9	2·0 22·7)	1	1·1
14	♂	8·25	4·8 (57·8)	3·5 (42·2)	0·8 (9·5	2·7 32·7)		1	0·9
15	♀	8·35	4·8 (57·6)	3·6 (42·4)	0·8 (9·0	2·8 33·4)		Small amount of second component	1·2
16	♀	8·50	5·4 (63·1)	2·9 (36·9)	0·7 (8·2	0·9 10·8	1·5 17·9)	2	0·85

* Mean values from ascending and descending patterns. The figures in brackets are percentages of the total protein.

The remarkable sudden increase in the serum proteins at the time of the development of a tumour and the fact that the increase is independent of the size of the tumour seem to exclude the possibility that the increased serum protein has its source in the disintegration of tumour tissue. This sudden increase in the serum proteins has an analogy with the reaction of the body to foreign protein, where the amount of antibody produced is largely independent of the amount of antigen present. An extensive literature is centred around the hypothesis that the proteins of tumours are antigenetically different from those of the host (Stern and Willheim, 1943). The present experiment seems to lend some support to such opinions. The drop in the serum protein of very ill animals also has its analogy with the drop of immunity in the terminal stages of infections. The results would suggest that, at least in hepatic tumours, the first pathological change in the serum proteins is an increase in the globulins, and a decrease is only the expression of the general deterioration of the diseased individual

The general impression that the serum protein of patients suffering from malignant diseases is decreased may be erroneously gained from reports (*a*) giving

mean values only, (*b*) not stating the localization of tumours, and (*c*) disregarding the general condition of the patient. Even in the recent report of Seibert *et al.* (1947), who give the mean values from 23 " cancer patients," the average total serum protein was low—6·6 g./100 ml.—but the values ranged up to 8·1 g./100 ml. Gray and Barron (1943) report individual analyses of sera from patients with hepatic tumours. Two out of 5 cases had high total proteins (8·99 and 8·38), two low (6·51 and 5·81), and one case was in the higher range of the normal (7·58). The clinical states of the patients were not mentioned. In an unpublished series of our own observations on the serum proteins of patients undergoing X-ray treatment, normal serum proteins were found in 25 mammary cancer cases, operated or non-operated (mean 7·19, range 6·5–8·0 g./100 ml.). High proteins were noted in two cases of pulmonary tumours (8·6 and 8·4 g./100 ml.) at the beginning of hospitalization, and in both cases the serum protein fell to a low level simultaneously with the clinical deterioration of the patient, reaching values between 5·7 and 5·9 g./100 ml. It seems possible that these findings may explain the discrepancies between the reports on the serum protein concentration of tumour patients by different investigators.

The non-homogeneity of the albumin in normal rat sera at pH 8 has not been previously reported. Electrophoretic analyses of rat sera have been made by Moore (1945) and by Deutsch and Goodloe (1945). These workers used veronal buffer of pH 8·6 and phosphate buffer of pH 7·4 containing 0·15 M NaCl. These conditions do not approximate sufficiently to those of our experiments to allow comparison.

Moore (1945) found none or very little α-globulin in the serum from rats of the Long-Evans strain. But this component was present after thyroidectomy (Moore, Levin and Smelser, 1945). Alpha-globulin was present in serum from our normal rats (blackhooded and Wistars), as well as from rats bearing hepatic tumours. The increase of the γ- and β-globulins of these rats might be due to an increase in immune bodies frequently associated with these fractions (Tiselius and Kabat, 1939). This explanation would be in accordance with the concept that the increase of the total serum protein in rats developing hepatic tumours is an immunological phenomenon. On the other hand, an increase of the serum lipids, likely to occur when the liver function is impaired, might cause, or contribute to, an increase of refractive area of the globulins. Since other hepatic or systemic diseases of rats were not investigated, we are not in a position to decide whether the changes in the serum proteins are an expression of malignant disease or are due to liver damage generally.

SUMMARY.

The serum proteins of rats fed pDA increase rapidly at the time of the manifestation of the tumour.

The increase of serum proteins is independent of the size of the tumour. The serum proteins drop to low levels when the health of the animals deteriorates.

In the patterns of the normal rat sera electrolysed in phosphate at pH 8·0 and μ0·1, α-, β- and γ globulin regions could be distinguished, and the albumin showed two components.

The rats which developed tumours due to feeding pDA showed a relative and absolute increase in the globulins, mainly in the β- and γ globulins. The concen-

tration of albumin was unchanged, but generally the albumin peak was not resolved into two components.

The possibility of the changes in the serum proteins being an immunological reaction is discussed.

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THE EFFECT OF 1:2:5:6-DIBENZANTHRACENE ON THE ASCORBIC ACID CONTENT OF THE LIVER OF RATS MAINTAINED ON HIGH AND LOW PROTEIN DIETS.

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KENNAWAY, Kennaway and Warren (1944) found that injection of certain carcinogenic substances, including 1:2:5:6-dibenzanthracene, caused an increase in the concentration of ascorbic acid in the liver of mice, whilst some non-carcinogenic, chemically related substances (naphthalene, anthracene, phenanthrene) did not show this effect.

In an investigation of the growth-inhibitory action of the carcinogenic hydrocarbon 1:2:5:6-dibenzanthracene (Elson and Warren, 1947) it was found that the inhibition of rat body growth and of the growth of Walker Rat carcinoma 256 (Elson and Haddow, 1947) by this substance is dependent on the protein content of the diet of the animals. On a 20 per cent protein diet one intraperitoneal injection of 500 mg./kg. 1:2:5:6-dibenzanthracene in the rat usually results in little growth inhibition for about the first 14 days, after which the animals at varying intervals lose weight and die. On a 10 per cent protein diet after the same dose of the carcinogen, immediate inhibition of growth occurs and persists for a prolonged period, usually until the death of the animal.

In considering the cause of the rise in liver ascorbic acid brought about by 1:2:5:6-dibenzanthracene, it appeared possible that this was a result of the growth-inhibitory action of the compound. There was some evidence (Elson, unpublished communication) that the growth inhibition was accompanied by a fall in the succinic dehydrogenase component of the succinoxidase enzyme system of the liver. Since the cytochrome oxidase component of this enzyme system is capable of rapid oxidation of ascorbic acid, it seemed possible that the rise in ascorbic acid after 1:2:5:6-dibenzanthracene might be the result of less destruction by such oxidation occurring owing to the decreased content of the oxidizing enzyme. The dependence of the growth-inhibiting action of the carcinogen on diet offered an opportunity for investigation of this idea, since if the suggestion that the rise in liver ascorbic acid is a consequence of the growth inhibition is correct, it would not be expected to occur in animals maintained on the 20 per cent protein diet until after the animal had begun to lose weight. Estimations of liver ascorbic acid have been carried out in rats maintained on 20, 10 and 5 per cent protein diets and treated with 1:2:5:6-dibenzanthracene.

EXPERIMENTAL.

Estimation of ascorbic acid.

This was carried out by the method described previously by Kennaway *et al.* (1944).

Diets.

The 20 per cent and 10 per cent protein diets were the same as those described by Elson and Warren (1947), and the 5 per cent protein diet the same as that described by Elson, Goulden and Warren (1947). The dry diets were made into a " dough " by mixing with a little water. The animals were kept in separate cages and fed amounts of diet in excess of their daily consumption. They were weighed every 2 or 3 days.

High and low protein tablets.

In later experiments it was found necessary to estimate the amount of food consumed by each rat, and in order to facilitate this 25 per cent and 5 per cent protein diets were made up in 5 g. tablets in the shape of cylinders of approximately 3 cm. diameter and 0·5 cm. high. This necessitated replacement of some of the starch in the original diets by cane sugar and glucose in order to obtain a firm tablet. The composition of these tablets is given in Table I.

TABLE I.—*Composition of 25 per cent and 5 per cent Protein Tablets.*

	Percentage Composition. Protein 25 per cent.	Protein 5 per cent.
Casein (light white—Glaxo Laboratories Ltd.)	25	5
Starch (maize)	18	34
Sucrose	30	42
Glucose	16	8
Margarine	5	5
Bemax (Vitamins Ltd.)	2·5	2·5
Salt mixture (Glaxo Laboratories Ltd.)	1	1
Chalk	0·5	0·5
Cod liver oil	1	1
Vitamin concentrate (aneurin 0·06%, riboflavin 0·4% in aqueous solution)	1	1
	100	100

The daily ration of each rat was usually 3 tablets (15 g.), and by weighing the remains the next day the food consumption could be determined with a fair degree of accuracy, since the tablets were found not to change appreciably in weight after exposure in air, and very little loss by crumbling occurred during eating.

1:2:5:6-Dibenzanthracene for injection was dissolved at a concentration of 50 mg. per c.c. in arachis oil by heating. The solution was cooled quickly whilst stirring with a glass rod, and the resulting mixture of partly dissolved and partly suspended dibenzanthracene used for intraperitoneal injection.

RESULTS.

The effects of 1:2:5:6-dibenzanthracene (500 mg./kg. i.p.) on the liver ascorbic acid of rats maintained on 20, 10 and 5 per cent protein diets are shown in Tables II, III and IV. From the control experiments, in which only arachis oil was injected, it is seen that the variation of the protein content of the diet itself has practically no influence on the level of ascorbic acid in the liver. The mean values are 231 μg. per g. liver for the animals maintained on the 20 per cent protein diet, 209 μg. for those on the 10 per cent protein diet and 222 μg. for those on the 5 per cent protein diet. The injection of dibenzanthracene causes an increase in the mean values for ascorbic acid content of 40 per cent (324 μg.

TABLE II.—*Ascorbic Acid Content of the Liver of Rats Maintained on 20 per cent. Protein Diet and Treated with 1:2:5:6-Dibenzanthracene (D.B.A.).*

Days after injection.	Body weight (g.).	Liver weight % of body weight.	Ascorbic acid μg per g. liver.
A. *Controls (1 c.c. arachis oil.)*			
7	105	6·44	224
7	109	6·72	203
7	120	6·0	230
10	145	5·0	278
10	153	5·46	238
10	146	5·24	215
Mean	130	5·81	231
B. *D.B.A. (500 mg./kg. in 1 c.c. arachis oil).*			
7	98·5	6·9	264
7	121	7·7	262
7	139	5·6	385
7	169	5·7	380
7	139	6·9	367
10	158	7·65	310
10	138	5·9	300
Mean	137	6·34	324

per g. liver) in the 20 per cent protein diet animals, 66 per cent (349 μg. per g. liver) in the 10 per cent protein diet animals, and 21 per cent (269 μg. per g. liver) in those maintained on the 5 per cent protein diet.

From Figure 1 it is clear that in the animals maintained on the 20 per cent protein diet the liver ascorbic acid had already increased 7 days after injection and before the growth inhibition occurred; also, the succinic dehydrogenase activity (Thunberg technique) of the livers of rats No. 1, 2 and 3 was found not to be significantly lower than that of control rats maintained on the same diet and injected with arachis oil only.

Referring again to Tables II, III and IV it is seen that the maximum increase in liver ascorbic acid caused by 1:2:5:6-dibenzanthracene occurs in the animals

maintained on the 10 per cent protein diet. It has been suggested (Elson and Warren, 1947) that the actual growth-inhibiting agent is a metabolic product of dibenzanthracene, and that the rate of absorption and metabolism of the carcinogen is thus related to the total metabolism of the animal, which is, in turn, controlled by the level of protein in the diet. Thus a dynamic equilibrium exists between the rate of production of the toxic metabolite and the total metabolism

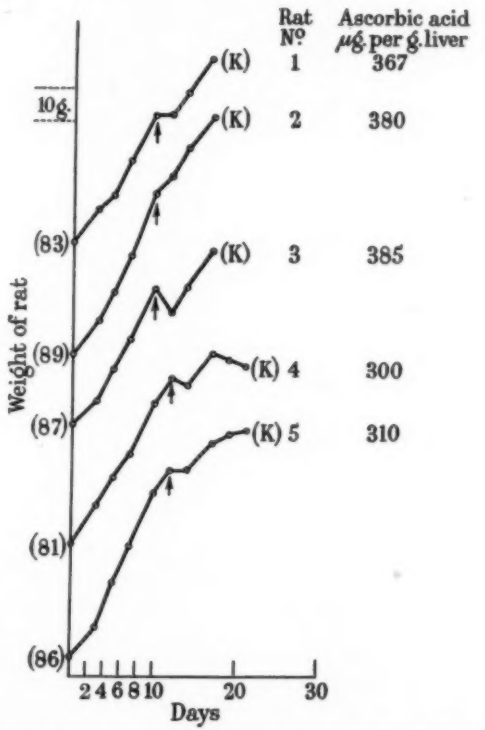

FIG. 1.—Growth rates of individual rats maintained on 20 per cent protein diet before and after treatment (shown by arrow) with 1:2:5:6-dibenzanthracene (500 mg./kg.), and ascorbic acid contents found for the liver of each rat when killed (K). Figures in parentheses give the initial weight of the rat.

of the animal. Judging by the magnitude of the growth-inhibiting effect of dibenzanthracene, this equilibrium appears to reach a maximum in the animals maintained on a diet of about 10 per cent protein content. The animals fed the 5 per cent protein diet grow only very slowly, but the effect of dibenzanthracene on them is not to make them lose weight, as would be expected if the magnitude of the growth inhibition was proportionate to that observed in animals maintained on the 10 per cent protein diet. This smaller effect is presumably related to the lower total metabolism of the 5 per cent protein diet animals, and

TABLE III.—*Ascorbic Acid Content of the Liver of Rats Maintained on* 10 *per cent Protein Diet and Treated with* 1:2:5:6-*Dibenzanthracene* (*D.B.A.*).

Days after injection.	Body weight (g.).	Liver weight % of body weight.	Ascorbic acid µg per g. liver.
A. *Controls* (1 *c.c. arachis oil*).			
8	142	4·24	280
8	120	3·82	250
11	120	5·57	192
12	108	4·46	210
14	139	4·55	149
15	167	4·63	171
Mean	133	4·55	209
B. *D.B.A.* (500 *mg./kg. in* 1 *c.c. arachis oil.*)			
8	115	6·52	374
8	122	5·8	374
11	129	5·8	313
12	120	6·5	357
13	107	6·8	384
14	161	6·2	318
15	136	7·7	325
Mean	127	6·47	349

it must be supposed that the smaller increase in liver ascorbic acid in these animals is also connected with the same cause.

If the growth-inhibiting effect of dibenzanthracene is caused by inhibition of protein synthesis, it seems possible that energy normally used for protein synthesis could, in the inhibited animals, be diverted to increased carbohydrate metabolism, and that the increase in liver ascorbic acid is associated with this increase. Observations of rats treated with growth-inhibiting carcinogens indicated no marked decrease in food consumption corresponding with the lower growth rate, and the animals usually appear very active in movement, etc.

Experiments were carried out in which the daily food consumption of individual rats was measured, before and after treatment with 1:2:5:6-dibenzanthracene. The food in these experiments consisted of the 25 per cent and 5 per cent protein tablets previously described (Table I). The results are given in Fig. 2 and 3. The growth rate of the rats fed on the 25 per cent protein tablets before treatment with dibenzanthracene was not as high as is usually obtained when the animals are fed the ordinary 20 per cent protein diet (Fig. 1), probably because less of the " tablet " food was consumed. This was reflected in the reaction of the rats to the dibenzanthracene.

Instead of producing no immediate effect on the growth rate, as in the case of those animals fed 20 per cent protein diet (Fig. 1), a considerable growth inhibition was observed. Immediately following the injection there was a drop in food intake lasting for one or two days, followed by rapid recovery of appetite

TABLE IV.—*Ascorbic Acid Content of the Liver of Rats Maintained on 5 per cent Protein Diet and Treated with 1:2:5:6-Dibenzanthracene (D.B.A.).*

Days after injection.	Body weight (g.).	Liver weight % of body weight.	Ascorbic acid μg per g. liver.
A. *Controls* (1 *c.c. arachis oil*).			
7	100	5·61	267
7	96	7·15	227
8	91	3·70	230
8	103	5·92	220
11	116	5·0	177
13	115	6·86	250
14	108	4·33	254
15	114	4·49	145
Mean	105	5·38	222
B. *D.B.A.* (500 *mg./kg. in* 1 *c.c. arachis oil*).			
7	100	7·0	251
7	76	6·5	294
7	75	6·36	315
7	96	7·15	305
8	102	6·0	253
11	111	7·6	252
14	85	7·15	236
15	118	4·81	292
15	116	6·8	195
15	109	5·8	298
Mean	99	6·52	269

(Fig. 2). The rats then reacted either by assuming an apparently prolonged lower rate of food intake (rat No. 1 and 2) which was accompanied by a rapid loss of weight, or the food intake remained at very little below the pre-injection level, although complete inhibition of growth and even loss of weight occurred (rats No. 3 and 4). In the case of rat No. 5, although complete inhibition of growth occurred, and at one time actual loss of weight, the food intake, after the initial drop, rose steadily to a value higher than the level before administration of dibenzanthracene

At the time the animals were killed for determination of liver ascorbic acid the actual degree of growth inhibition was greater than the maximum previously obtained with the 10 per cent protein diet animals. The level of liver ascorbic acid was also the highest obtained (mean 386 γ per g. liver).

The rats maintained on the 5 per cent protein tablets also showed a drop in food intake immediately following the injection of 1:2:5:6-dibenzanthracene, but soon regained their initial level (Fig. 3). This level was then retained or in some cases slightly increased for the duration of the experiment. The effect of the dibenzanthracene on the growth rate of the animals was not nearly as great as with the animals fed the 25 per cent protein tablets, and the level of liver ascorbic acid was also not so high (mean 344 γ per g. liver).

DISCUSSION.

Rats maintained on a 20 per cent protein diet show an increase in liver ascorbic acid within 7 days of injection of 1:2:5:6-dibenzanthracene, before any marked inhibition of growth or decrease in liver succinoxidase occurs. This does not support the idea that the rise in liver ascorbic acid results from a decreased

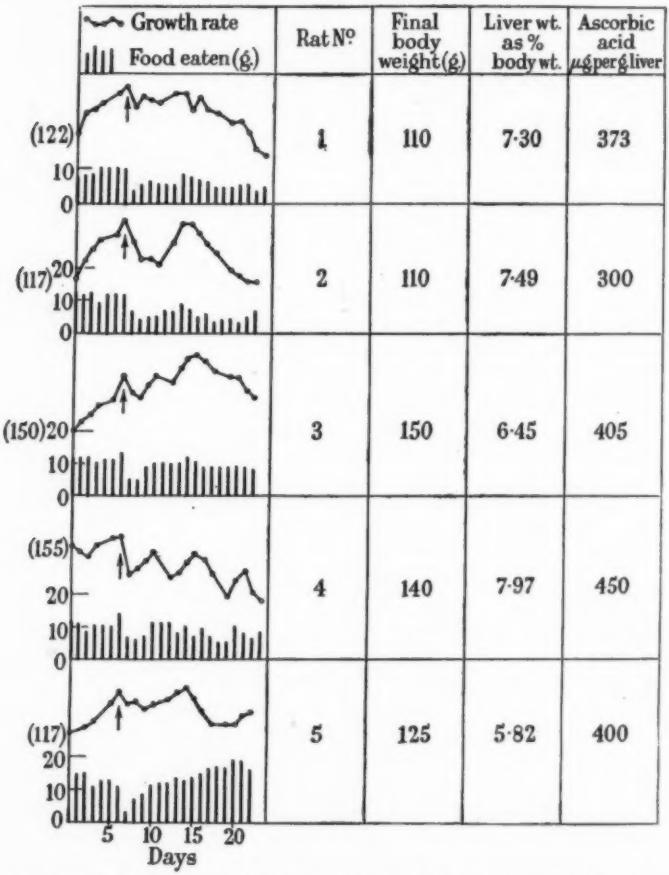

FIG. 2.—Growth rate, food intake, and liver ascorbic acid of rats maintained on 25 per cent protein tablets and injected (shown by arrow) with 1:2:5:6-dibenzanthracene (500 mg./kg. i.p.). Figures in parentheses give the initial weight of the rat.

destruction of the vitamin owing to the lowered level of oxidizing enzymes, being thus an indirect effect of the growth-inhibitory action of the carcinogen.

Comparison of the magnitude of the increase in liver ascorbic acid caused by dibenzanthracene in rats maintained on 20, 10 and 5 per cent protein diets

suggests rather that the increase is roughly directly proportional to the amount of growth inhibition obtained. The degree of growth inhibition is dependent on an equilibrium being established between the rate at which the animal is metabo-

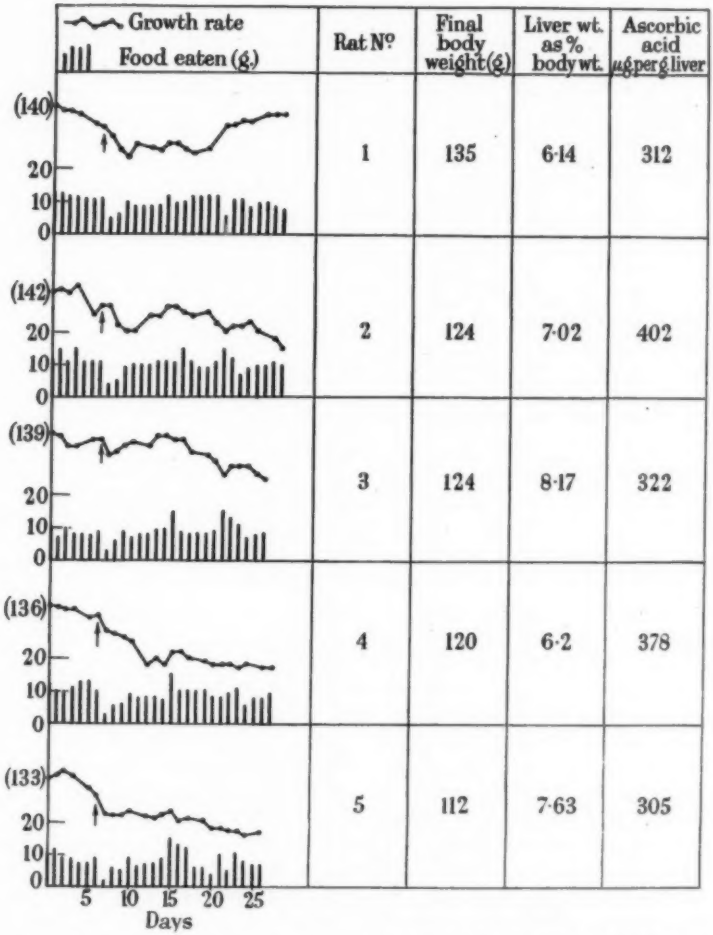

FIG. 3.—Growth rate, food intake, and liver ascorbic acid of rats maintained on 5 per cent protein tablets and injected (shown by arrow) with 1:2:5:6-dibenzanthracene (500 mg./kg. i.p.). Figures in parentheses give the initial weight of the rat.

lizing the hydrocarbon and the rate at which it is synthesing protein necessary for growth. In animals maintained on a 20 per cent protein diet there is a rapid rate of general metabolism, and hence presumably of metabolism of the dibenzanthracene to a "toxic" metabolite which exerts its growth-inhibitory action

by interference with protein synthesis. In these animals, however, the high protein diet ensures an ample supply of all amino-acids needed for synthesis of tissue proteins, and thus, because of the rapid rate of protein synthesis, the effect of the inhibitor is not immediately apparent, and the animal continues to grow until accumulation of toxic metabolite eventually results in rapid loss in weight followed by death (Elson and Warren, 1947). In animals maintained on low protein diets, although the rate of production of the " toxic " metabolite may not be so high, the animals' rate of protein synthesis is already restricted by the limited supply of amino-acids in the diet, and on treatment with dibenzanthracene an immediate effect on growth is observed.

It seems possible, therefore, that the increase in liver ascorbic acid in rats treated with dibenzanthracene may be directly related to the inhibitory action of the substance on protein metabolism and synthesis. In these animals energy normally used for protein synthesis may be diverted to increased carbohydrate metabolism, and the rise in liver ascorbic acid may be associated with this increase. The assessment of the amount of food eaten following injection with the carcinogen lends some support to this idea. After an initial short period of loss of appetite the animal may react by assuming a somewhat lower level of food intake, or may continue to eat much the same or an even greater amount of food daily, although growth may be completely inhibited.

Under these latter conditions a very profound change in the basal metabolism of the animal must presumably have taken place, and considerable investigation will be necessary before the significance of this change can be fully assessed. At present, pending further investigation, it can only be suggested that the rise in liver ascorbic acid may well be one of the results of this altered metabolism.

SUMMARY.

The increase in liver ascorbic acid produced by treatment with 1:2:5:6-dibenzanthracene in rats maintained on 20, 10 and 5 per cent protein diets has been studied. In animals fed 20 per cent protein diet liver ascorbic acid increases before any marked growth-inhibition is observed, and the increase is thus not an indirect consequence of the inhibition of growth.

The growth-inhibition is not merely the result of a lowered intake of food.

The animals may react to treatment with dibenzanthracene by maintaining or even increasing their daily food consumption, although growth is completely inhibited. This suggests that a profound change in basal metabolism must have taken place, and the rise in liver ascorbic acid may be associated with this change.

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EXPERIMENTS ON THE IDENTIFICATION OF 3:4-BENZPYRENE IN DOMESTIC SOOT BY MEANS OF THE FLUORESCENCE SPECTRUM.

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THE carcinogenic activity of ethereal extracts of domestic soot was demonstrated by Passey (1922). Domestic soot may contain as much as 40 per cent of tarry matter (Cohen and Ruston, 1925). The use of the fluorescence spectrum by Mayneord and Hieger led to the identification of 3:4-benzpyrene in gas-works pitch (Cook, Hewett and Hieger, 1933). This discovery suggests that this hydrocarbon might be one of the carcinogenic agents in domestic soot, although the conditions of pyrolysis in the domestic hearth, and in the gas-works retort, are different. Berenblum and Schoental (1947) found that carcinogens other than 3:4-benzpyrene were present in coal tar. The first attempt to identify 3:4 benzpyrene as a contaminant of the atmosphere was made by Hieger (1946), who exposed non-fluorescing benzene to the air in London, and found that the fluid became fluorescent and showed spectral bands resembling those of benzpyrene. Kennaway and Kennaway (1947) as a result of their investigations of the incidence of cancer of the lung suggested that "The higher mortality from cancer of the lung in towns (Stocks), the low mortality in agricultural occupations, and the absence of a social gradient (Stevenson), are compatible with an aetiological factor in the air such as coal smoke."

In view of this suggestion, and in consideration of the fact that domestic soot is the chief atmospheric contaminant, a search for 3:4-benzpyrene in soot was undertaken.

MATERIALS AND METHODS.

The apparatus included a Hilger Raman spectrograph, Ilford Zenith plates and a mercury vapour lamp (Kelvin, Bottomley and Baird), which was housed in a metal box fitted with a Wood's Glass window. The solutions were contained in thin-walled Monax Glass tubes of 8 mm. diameter. The exposure required was 2 minutes with a slit width of 0·125 mm. The plates were developed according to the time and temperature table supplied with the developer. Under these conditions a solution of 0·1 μg. of benzpyrene per ml. of petroleum ether gave a reproducible spectrum that was just visible on the photographic plate.

Reagents.

Benzene, non-fluorescent.—Pure benzene washed three times with concentrated sulphuric acid, dried and distilled.

Analar petroleum ether (b.p. 60°–80°).

Spence Type H. alumina.—Activated alumina loses activity when exposed to air. The absorptive power of a particular sample appeared to be correlated with the pH of a suspension in distilled water. For the present purpose, samples showing a pH value of 10·0–10·3 were found to be most suitable.

3:4-*Benzpyrene.*—The commercial product contains a pale-blue-fluorescing impurity which is very strongly adsorbed on alumina. The sample used was purified by absorption on alumina, elution with benzene and recrystallization. Stock solution of benzpyrene : 10 mg. dissolved in 100 ml. benzene. Dilute solution, 1 μg./ml. : 1 ml. of stock solution diluted to 100 ml. with petroleum ether. Standard solutions were prepared by diluting 8, 6, 4, 2 and 1 ml. respectively to 10 ml. with petroleum ether. The solutions appeared to be quite stable.

The first attempts to separate benzpyrene from soot were based on the method described by Berenblum (1945) for the extraction of benzpyrene from coal tar. In the original method vacuum distillation of the suitable fraction of tar was followed by solution of the distillate in concentrated sulphuric acid. Attempts at vacuum distillation of benzene extracts of soot showed, however, that the composition of the soot extract was very different from that of coal tar. Evaporation of the benzene extract yielded a residue consisting mainly of non-volatile material, which appeared to decompose on distillation *in vacuo* with evolution of gas.

Direct sulphuric acid extraction of the crude tar extracted from soot was also unsuccessful, probably owing to considerable sulphonation. Moreover, recovery experiments, with microgram quantities, of pure benzpyrene from ice-cold sulphuric acid showed that considerable loss of the hydrocarbon occurred, and that the method was useless as a quantitative one for such small amounts.

Chromatographic adsorption was next tried. When an extract of soot was submitted to chromatographic separation, the characteristic fluorescence spectrum of benzpyrene was given by the appropriate eluate. Experiments with various samples of alumina showed that Spence's Type H alumina, containing adsorbed benzpyrene, could be washed freely with a mixture of benzene-petroleum ether (1:10) without elution, and that the benzpyrene was eluted only when the proportion of benzene was increased (3:10). Some fluorescent impurities remained in this eluate, but by suitable dilution with petroleum ether, spectra could be obtained showing benzpyrene bands reasonably free from background (Fig. 4). The benzpyrene content was then estimated by visual comparison with a set of standards (Fig. 1), photographed and developed under the same conditions (Berenblum and Schoental, 1942). Experiments upon photometric comparison are in progress.

The details of a typical experiment are as follows :

1 g. of mixed domestic soot (from a chimney sweep's stock) was extracted in a Soxhlet apparatus. It was found that by refluxing for about 5 hours with 100 ml. of a 1:1 mixture of benzene-petroleum ether, the benzpyrene could be

DESCRIPTION OF PLATES.

FIGS. 1–4.—Identification of 3:4-benzpyrene by means of the fluorescence spectrum.
FIG. 1.—3:4-Benzpyrene standards in petroleum ether.
FIG. 2.—Soot eluates of increasing benzene content.
FIG. 3.—Comparison of soot eluates 4 and 5 with benzpyrene.
FIG. 4.—Petroleum ether dilutions of eluate 4 for comparison with standard.

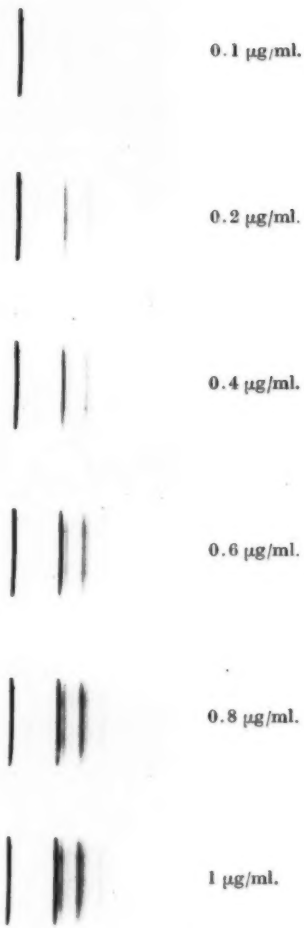
0.1 μg/ml.
0.2 μg/ml.
0.4 μg/ml.
0.6 μg/ml.
0.8 μg/ml.
1 μg/ml.

Fig. 1.

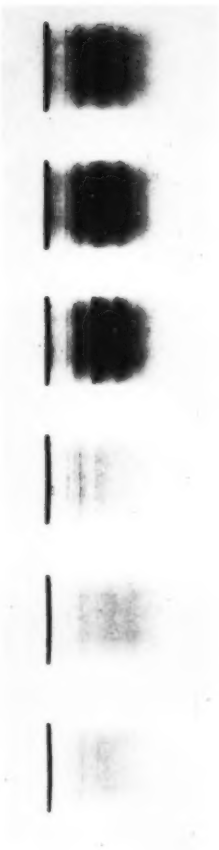
Eluate 6.
50% benzene.
Eluate 5.
30% benzene.
Eluate 4.
30% benzene.
Eluate 3.
10% benzene.
Eluate 2.
10% benzene.
Eluate 1.
petroleum ether.

Fig. 2.

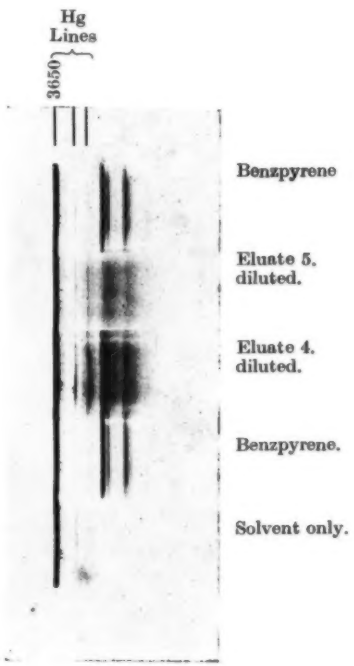
Hg
Lines
3650
Benzpyrene
Eluate 5.
diluted.
Eluate 4.
diluted.
Benzpyrene.
Solvent only.

Fig. 3.

Benzpyrene.
0.1 μg/ml.
Dilution
1:50
2:50
3:50

Fig. 4.

extracted without some part of the other hydrocarbons present. The material extracted amounted to 18 per cent of the soot. The extract was given a preliminary purification by passing it through a short column of alumina (14 × 25 mm.) and eluting with the same mixture of solvents. The solvent was then distilled off, the residue dissolved in 20 ml. of petroleum ether and adsorbed on a column of alumina (14 × 110 mm.). On developing with petroleum ether two purple bands were visible under ultra-violet light. The lower band was easily eluted with benzene-petroleum ether (1:10), while the upper could only be washed down when the benzene content of the eluting fluid was increased (3:10). This latter fraction is shown in Fig. 2, as Eluate 4, total volume 58 ml. Continued elution with the same solvent resulted in an eluate (Fig. 2, Eluate 5), which on dilution (Fig. 3) gave a spectrum no longer identical with that of benzpyrene (Fig. 3, Eluate 5 diluted). The difference is not due to any change of solvent (Chalmers, 1938), as the eluting and diluting fluids for (4) and (5) were the same. Fifty-fold dilution of the 4th eluate (Fig. 4) gave a spectrum in which the intensity of the bands approximated to that of a benzpyrene standard containing 0·1 μg./ml. Since this was the only fraction containing benzpyrene, the benzpyrene content of the original soot appeared, by this method, to be 290 mg./kg., or 0·029 per cent.

A series of analyses upon the same sample of domestic soot are shown in Table I.

TABLE I.

Mixed domestic soot from chimney sweeps stock.	3:4-Benzpyrene (%).
Preliminary analyses	0·045
	0·027
	0·030
	0·024
Technique finally adopted	0·029
	0·025
Mean	0·030

The results are consistent with one another, and experiments on the absolute quantitative accuracy are in progress.

SUMMARY.

1. 3:4-Benzpyrene has been identified by means of fluorescence spectroscopy as a component of tar obtained by extraction of domestic soot with a benzene-petroleum ether mixture.

2. Adsorption of the extracts on alumina and fractional elution with benzene-petroleum ether yielded solutions suitable for tests of quantitative recovery of benzpyrene by measurement of its fluorescent spectrum.

3. A mixed sample of domestic soot showed a benzpyrene content of about 300 mg./kg. when examined by this method.

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SURVEY OF PAPERS.

ATKIN, FENNING, HEADY, KENNAWAY AND KENNAWAY find from a study of death certificates of cases of cancer of the skin and lip in certain occupations that the mortality is highest in agricultural workers, lowest in the professional classes, while miners take an intermediate position. The data presented indicate that other factors besides exposure to light are involved in the causation of cancer of the skin and lip (p. 1).

JAPHA reviews the literature on the treatment of glioma retinae, and describes the history, pathology and treatment of 19 cases seen at the Middlesex Hospital (p. 16).

JOLLES describes a method of studying direct and indirect effects of irradiation by obtaining material for histological examination from accessible tumours irradiated through a lead sieve with square apertures (p. 27).

DALE, DAVIES AND MEREDITH describe experiments on the inactivation by X-rays of biologically active compounds in aqueous solution through some change in the solvent, which in turn acts on the solute, and the reduction of this effect by the presence of a second solute (p. 31).

BURCHELL, EARLE AND MACLAGAN find that an increase in urinary cholesterol occurs in about 10 per cent of cases of cancer after allowance is made for increases in cholesterol due to associated albuminuria or pyuria (p. 42).

BURCHELL AND MACLAGAN report experiments which support the view that the urinary cholesterol is normally associated with a urinary proteose fraction and also with heat-coagulable protein if present. They describe a method of estimation which avoids most of the disadvantages of those previously described (p. 52).

ROBINSON AND GOULDEN discuss methods for the chromatographic separation and estimation of urinary 17-ketosteroids and describe a technique which yields reproducible results. In normal males the amounts of androsterone and of aetiocholanolone are approximately equal, but in prostatic disease the latter substance is present in relative excess (p. 62).

CAMPBELL has studied the ectodermal proliferation in the chorio-allantois that occurs in association with the growth of tumours implanted on the chorioallantoic membrane of the chick embryo. It is suggested that tumours may contain growth-promoting substances capable of causing the overgrowth of the ectodermal cells (p. 72).

BEGG finds that the intranasal administration of milk factor derived from sporadic mammary cancers can induce mammary tumours in hybrid mice free of the milk factor (p. 88).

GORER AND LAW describe an attempt to detect protective antibodies against the milk agent in the serum of mice. They have obtained some evidence of neutralisation with heated serum from mice of a higher cancer strain, but none with the other mouse sera tested (p. 90).

COWEN finds that the injection of pentose nucleotides almost halves the number of lung tumours induced in mice by the administration of urethane. It is suggested that pentose nucleotides may neutralise the leucopaenic action of urethane or supplement the normal cell purines, and thus reverse the carcinogenicity of urethane whether it acts through a virus or similar organism or by a metabolic upset (p. 94).

STRONG discusses the evidence in favour of the concept that cancer arises from a process of somatic mutation (p. 97).

BERENBLUM AND SHUBIK find that the total number of tumours induced on the skin of mice by a single application of a carcinogen followed by repeated paintings with croton oil is directly proportional to the concentration of the carcinogen. The known mutagenic agent, mustard gas, has failed to induce tumours under the same conditions. The results are discussed in relation to the somatic mutation theory of cancer causation (p. 109).

BOYLAND AND HORNING have produced tumours in 10 of 14 mice by the weekly subcutaneous injection of nitrogen mustards. They suggest that the nitrogen mustards act by interfering with mitosis, and thus increase the chances of somatic mutation from normal to malignant cells occurring (p. 118).

HIEGER has obtained 69 sarcomas at the site of injection of lipoid substances in approximately 2000 mice. Sixty-three of the tumours have been produced by unsaponifiable fractions from human and animal tissues, suggesting that the carcinogenic factor is cholesterol or a combination of cholesterol and a small proportion of a frequently occurring co-carcinogen (p. 123).

HOCH-LIGETI, HOCH AND GOODALL find that the serum globulins in rats fed *p*-dimethylamino-azobenzene increase rapidly during the development of hepatic tumours. The possibility of the changes in the serum proteins being an immunological reaction is discussed (p. 140).

ELSON, KENNAWAY AND TIPLER have studied the increase in liver ascorbic acid produced by injections of dibenzanthracene in rats maintained on high and low protein diets. The increase in ascorbic acid takes place before growth-inhibition, which may occur without lowered intake of food (p. 148).

GOULDEN AND TIPLER have identified 3:4-benzpyrene in tar obtained by extraction of domestic soot by fluorescence spectroscopy (p. 157).